

Regulation of Endocytosis and the Lysosome System in Skeletal Muscle

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This dissertation is based on the following papers which in the text will be referred to by their Roman numerals.

- I. Sellin LC, Libelius R, Lundquist I, Tågerud S and Thesleff S (1980) Membrane and biochemical alterations after denervation and during reinnervation of mouse skeletal muscle. *Acta Physiol. Scand.* 110:181-186.
- II. Tågerud S and Libelius R (1984) Lysosomes in skeletal muscle following denervation. Time course of horseradish peroxidase uptake and increases of lysosomal enzymes. *Cell Tissue Res.* 236:73-79.
- III. Libelius R and Tågerud S (1984) Uptake of horseradish peroxidase in denervated skeletal muscle occurs primarily at the endplate region. *J. Neurol. Sci.* 66:273-281.
- IV. Tågerud S and Libelius R (1985) Receptor-mediated uptake of horseradish peroxidase in innervated and denervated skeletal muscle. *Exp. Cell Res.* 160:95-105.
- V. Tågerud S, Libelius R and Thesleff S. An ultrastructural study of the segmental uptake of horseradish peroxidase in the endplate region of denervated skeletal muscle fibres. *J. Neurol. Sci.* (accepted for publication).
- VI. Tågerud S, Jirmanová I and Libelius R. Biochemical and ultrastructural effects of chloroquine on horseradish peroxidase uptake and lysosomal enzyme activities in innervated and denervated mouse skeletal muscle. *J. Neurol. Sci.* (accepted for publication).
- VII. Tågerud S, Libelius R and Thesleff S. Effects of botulinum toxin induced muscle paralysis on endocytosis and lysosomal enzyme activities in mouse skeletal muscle. *Pflügers Arch.* (accepted for publication).

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INTRODUCTION

The work presented in this thesis represents a continuation of the studies on endocytosis and the lysosome system in skeletal muscle which were initiated in this department more than 10 years ago and which have already been the subject of one previous thesis (Libelius 1978).

Endocytic processes as well as the formation and functions of lysosomes have previously been studied in more detail in cells from tissues other than skeletal muscle. The introduction of these processes and functions will therefore rely to a large extent on data obtained from studies on cell types different from skeletal muscle fibres.

ENDOCYTOSIS

The term endocytosis refers to the mechanism whereby living cells take up macromolecules or particles which are too large to penetrate the cell membrane. The process involves trapping of material inside an infolding of the plasma membrane followed by a membrane fusion event which results in the formation of an intracellular vesicle. Uptake of large particles (0.5-1 μm , Mellman 1984) is usually referred to as phagocytosis (cell eating). The uptake of smaller particles (*e.g.* lipoproteins), macromolecules, fluid and low molecular weight solutes is referred to as pinocytosis (cell drinking). Pinocytosis occurs by the formation of small (0.1-0.2 μm diameter, Mellman 1984) pinocytic vesicles. In mammals phagocytosis is usually associated with specialized cells such as macrophages. Pinocytosis, on the other hand, appears to be a largely constitutive function of cells (Mellman 1984) and has been observed to take place in the vast majority of cells with the exception of bacteria and the mature red blood cells of mammals (Jacques 1969). Formation of pinocytic vesicles and hence uptake by pinocytosis is not detectable in mammalian cells at 4°C (Silverstein *et al.* 1977).

Fluid-phase pinocytosis

Fluid-phase pinocytosis refers to the uptake of an impermeable substance which does not bind to the plasma membrane. The uptake of such a substance occurs merely as a consequence of its presence in the extracellular fluid which enters the cell by the pinocytic mechanism. The rate of uptake of a substance which accumulates by fluid-phase pinocytosis should therefore vary linearly with the concentration of the substance in the extracellular fluid.

Adsorptive pinocytosis

Adsorptive pinocytosis refers to the uptake of a substance which binds to the plasma membrane. Such a substance is taken up both in the fluid phase and bound to the membrane of the pinocytic vesicles. Since the binding sites become saturated at high concentrations the uptake occurs in a concentration dependent manner. At low, non-saturating concentrations the rate of uptake will depend mainly on adsorption and will be very large compared to that of a substance taken up by fluid-phase pinocytosis. At higher concentrations, as the binding sites become saturated, the adsorptive component of the uptake becomes less significant and the rate of uptake will tend to become linearly related to the concentration as for a substance taken up by fluid-phase pinocytosis.

The part of the plasma membrane to which a substance binds has been called a receptor or a binding site. Such sites may be non-specific, accepting a wide range of molecules, or they may be specific for one molecule or group of molecules. Non-specific binding of substances may be due to electrostatic and/or hydrophobic interactions (Pratten *et al.* 1980). Specific binding has been described for a number of ligands which bind with high affinity to plasma membrane sites (receptors). The pinocytic uptake of such ligands is usually referred to as receptor-mediated endocytosis.

Receptor-mediated endocytosis

A large number of substances including carrier proteins (such as low density lipoprotein and transferrin), viruses, toxins, hormones, immunoglobulins and glycoproteins containing different terminal sugar residues, have been shown to be taken up into various cells by a mechanism of receptor-mediated endocytosis (see Neville and Chang 1978; Anderson and Kaplan 1983; Steinman *et al.* 1983). The receptors for such ligands occur in specialized regions of the plasma membrane or cluster in such regions before uptake (see Goldstein *et al.* 1979). In many cases the uptake of receptors is believed to occur continuously whether or not binding of any ligand has occurred (see Anderson and Kaplan 1983; Brown *et al.* 1983; Mellman 1984). The specialized regions of the plasma membrane where receptors cluster are called coated pits because of their indented configuration and the presence of a fuzzy cytoplasmic coat which is composed mainly of a single protein called clathrin. The clathrin coat is thought to function in selecting and excluding molecules from the coated pit and to provide a structural scaffold for the invagination of a vesicle (Pearse and Bretcher 1981). The coated vesicles so formed are short-lived structures which lose their coat within 15-60 s after their formation (see Steinman *et al.* 1983; Mellman 1984). The vesicles are then believed to fuse together, or fuse with a larger endocytic vacuole, to form the endosome.

Endosomes

Endosomes are often irregular in shape and vary in size from 0.2-1.0 μm (Mellman 1984). They are considered as pre-lysosomal compartments and may be the principal site of receptor recycling (see Steinman *et al.* 1983). Endosomes do not contain lysosomal enzymes but appear to be acidic organelles with an internal pH similar to that of lysosomes (Mellman 1984). Fusion with lysosomes is inhibited by temperatures $< 20^{\circ}\text{C}$ despite continued receptor-mediated endocytosis (Dunn *et al.* 1980).

Receptosomes

A different mechanism for receptor-mediated endocytosis has also been proposed (see Willingham *et al.* 1981; Willingham and Pastan 1984; Pastan and Willingham 1985). According to this model coated pits never pinch off from the membrane to form true intracellular coated vesicles. Instead, uncoated vesicles (receptosomes) are formed by invagination of membrane adjacent to the coated region. When first formed receptosomes measure about 0.2 μm in diameter but by fusing with each other they can produce vesicles up to 0.6 μm in diameter (Pastan and Willingham 1985). As for the endosomes described above, receptosomes do not contain lysosomal enzymes but have an acidic pH and are believed to be involved in the recycling of membrane receptors (see Willingham and Pastan 1984). A difference between receptosomes and endosomes is, however, that receptosomes are not believed to fuse directly with lysosomes but first fuse with a specialized region of the Golgi apparatus for sorting of vesicle content and membrane receptors (Willingham and Pastan 1984; Pastan and Willingham 1985).

Phagosomes

The term phagosome was first used by Straus (1958) for cytoplasmic granules with the ability to segregate protein molecules, and other materials of macromolecular or colloidal size, taken up into cells by endocytosis. In later studies (Straus 1964) it was shown that at early stages after treatment with horseradish peroxidase (HRP) newly formed phagosomes were separate from pre-existing lysosomes in the tubule cells of the kidney and in the Kupffer cells of the liver. These light microscopic studies were the first studies which showed that substances taken up by endocytosis pass through a pre-lysosomal compartment before reaching secondary lysosomes. The term phagosome has later been claimed to be inappropriate for the pre-lysosomal compartment since the vacuole does not arise by phagocytosis (Helenius *et al.* 1983; Mellman 1984). In later studies the pre-lysosomal vacuoles have been termed pinosomes, endosomes, intermediate vacuoles and receptosomes (Helenius *et al.* 1983).

In the ultrastructural studies included in the present work the term phagosome has been used to

describe a membrane-limited body larger than 0.2-0.3 μm and which accumulates HRP.

Membrane and receptor recycling

Quantitative studies on pinocytosis in mouse peritoneal macrophages have provided strong support for membrane recycling during pinocytosis (Steinman *et al.* 1976). These cells were shown to take up the equivalent of 26% of their cell volume and 190% of their cell surface area each hour. Despite this, cell volume and area remained constant as did the volumes and areas of pinocytic vesicle and secondary lysosome compartments.

Evidence in strong support of recycling of membrane interiorized by endocytosis has also been obtained in studies on cultured rat embryo fibroblasts (Schneider *et al.* 1979a; 1979b). In these studies fibroblasts were first allowed to store goat immunoglobulins directed against rabbit immunoglobulins. When cells were later exposed to rabbit immunoglobulins, known to bind to the cell membrane, a substantial portion of the stored goat immunoglobulins was unloaded from its intracellular storage site suggesting that the rabbit immunoglobulins bound to the membrane had been internalized, reached the intracellular compartment containing goat immunoglobulins and then recycled to the cell surface with some of the goat immunoglobulins bound.

Much evidence has also accumulated which strongly suggests that cells engaged in receptor-mediated endocytosis can internalize an amount of ligand which far exceeds their total number of receptors for that ligand. This uptake has also been shown to be independent of new receptor synthesis (see Brown *et al.* 1983; Steinman *et al.* 1983; Mellman 1984). The conclusion drawn from such studies is that the cells take up ligand bound to a limited number of receptors which are reutilized for several rounds of uptake.

The intracellular release of ligand is believed to occur in endosomes/receptosomes where the acidic pH may cause dissociation of receptor-ligand complexes (see Brown *et al.* 1983; Steinman *et al.* 1983; Mellman 1984; Maxfield 1985). The recycling of membrane and receptors without concomitant unloading of substances taken up by the endocytic process also requires a great deal of intracellular sorting of fluid, solutes and membrane. The details of these sorting mechanisms are largely unknown (see Mellman 1984; Willingham and Pastan 1984; Harding *et al.* 1985).

Endocytosis in skeletal muscle

Skeletal muscle is not a tissue which has been used very extensively for studies on endocytosis. It was noted, however, that the endocytic activity of this tissue could influence the estimated number of acetylcholine receptors when measured by ^3H - α -neurotoxin binding at 37°C (Libelius 1974; 1975). The binding of such a toxin to muscles at 37°C could be described by two different uptake mechanisms (Libelius 1978). One was a saturable binding to acetylcholine receptors which also occurred at 4°C and the other was a slow acetylcholine receptor independent uptake which

occurred linearly with time only at 37°C. It was further shown that the slow temperature sensitive α -neurotoxin uptake was higher in denervated than in innervated muscles (Libelius 1974; 1975).

Following these observations a number of studies were carried out in order to characterize the endocytic activity of skeletal muscle using the more conventional extracellular markers ^3H -inulin and HRP. These studies confirmed the early results showing that denervated muscles have an increased endocytic activity (Libelius *et al.* 1978a; 1979a; Boegman and Scarth 1981b). Neural application of batrachotoxin, which inhibits axonal transport and impulse activity in the nerve, was also shown to increase the endocytic activity of muscle fibres (Boegman and Scarth 1981b). In denervated muscles the increase in uptake of HRP was shown to occur only in limited segments of the muscle fibres (Libelius *et al.* 1979a).

The endocytic activity of muscle fibres has also been shown to increase if muscles are incubated in the presence of polycationic proteins such as protamine (Libelius 1975; Jirmanová *et al.* 1977; Libelius *et al.* 1978a).

In muscles from mice and chickens suffering from hereditary muscular dystrophy the endocytic activity has also been shown to be increased and ultrastructural cytochemistry showed a segmental localization of HRP uptake in the muscle fibres, as after denervation (Libelius *et al.* 1978b; 1979b).

In all the above conditions where an increase in endocytic activity of muscles has been shown, muscle lysosomal enzyme activities are also increased (see Weinstock and Iodice 1969; Libelius and Lundquist 1978). These observations resulted in a working hypothesis which suggested that increased endocytosis in skeletal muscle induces increased lysosomal enzyme activities and muscle fibre degeneration (see Libelius *et al.* 1978c; Thesleff *et al.* 1979; Thesleff and Libelius 1983).

LYSOSOMES

The word lysosome means lytic particle and was proposed as the name for particles isolated from rat liver homogenates by differential centrifugation and which contained a number of enzymes with latent hydrolytic activities with acidic pH optima (see de Duve 1969). The two biochemical criteria for defining a lysosome are that it contains several different acid hydrolases and that it is surrounded by a lipoprotein membrane (de Duve 1983).

Morphologically, lysosomes are also identified by the same two criteria: a surrounding membrane and the occurrence of one or more acid hydrolases. Since lysosomes vary much in size, shape and internal structure these criteria are not very useful for morphological identification. The morphological identification has therefore often required cytochemical staining for acid hydrolases, most often acid phosphatase. It has been pointed out, however, that there is a danger in extending the name lysosome to any intracellular structure staining positively for acid phosphatase (de Duve 1963) since the biochemical definition, and the functional implication of a concerted lytic action, requires the presence of a number of different acid hydrolases. It has to be

kept in mind that acid phosphatase activity may be present in some locations without the accompaniment of other hydrolytic enzymes and there exert a function which is unrelated to that of lysosomes. An example of this may be the cytochemical demonstration of acid phosphatase activity in transverse tubuli (t-tubuli) of avian skeletal muscle (Trout *et al.* 1979b; 1981a). Other morphological observations such as pinocytic labelling and indications that the structure examined is or has been the site of a concerted lytic activity (*e.g.* presence of dense deposits, membranous whorls or other materials with a "messy" appearance) may aid in the decision to apply the name lysosome to a given structure exhibiting acid phosphatase activity (de Duve 1963; 1983).

Functions of lysosomes

Lysosomes, because of their content of large numbers of different hydrolytic enzymes, have been implicated to take part in various different cellular digestive processes such as autophagy, crinophagy and the degradation of endocytosed materials (see *e.g.* de Duve 1969; Dean and Barret 1976). It has also been shown, however, that lysosomes are not necessarily a metabolic dead end from which trapped molecules can escape only in the form of small diffusible digestion products. From a continuation of the studies on uptake and release of different specific immunoglobulins in cultured rat embryo fibroblasts, referred to above (Schneider *et al.* 1979a; 1979b), it was concluded that the lysosomes are connected with the extracellular medium by means of mobile membrane patches acting as some sort of endless moving belt capable of transporting materials in both directions (Schneider *et al.* 1981). Lysosomes have also been suggested to play a role in the transport of certain hormones to the nucleus apparently without degradation of the hormones (see Dean and Barret 1976).

Formation of lysosomes

The polypeptide chains of lysosomal enzymes are synthesized on polysomes attached to the membrane of the rough endoplasmic reticulum. Carbohydrate chains are added to the polypeptides in the endoplasmic reticulum and in the Golgi apparatus where the carbohydrates are also further processed (see Marzella and Glaumann 1983; Erickson *et al.* 1984; Goldberg *et al.* 1984). Of particular interest is that mannose residues on several lysosomal enzymes have been shown to become phosphorylated in the endoplasmic reticulum or in the Golgi apparatus (see Sly and Fischer 1982; Marzella and Glaumann 1983; Shepherd *et al.* 1983; Goldberg *et al.* 1984). The mannose-6-phosphate so formed is used as a recognition marker for specific receptors which segregate the lysosomal enzymes from secretory glycoproteins in the Golgi apparatus (see Sly and Fischer 1982; Marzella and Glaumann 1983; Creek and Sly 1984; Jourdian *et al.* 1984). The lysosomal enzymes are then packed into small coated vesicles which pinch off from the Golgi apparatus. These vesicles then fuse with *e.g.* lysosomes or endocytic vacuoles to release their

content of hydrolytic enzymes. As for receptor-mediated endocytosis the release of enzymes from the receptors is believed to be facilitated by the low pH of the receiving structures (see Sly and Fischer 1982; Sly and Grubb 1984). Vesicles with mannose-6-phosphate receptors are then believed to recycle back to the Golgi apparatus in order to be reused for additional rounds of enzyme transport (see Sly and Fischer 1982; Jourdain *et al.* 1984). Other recognition markers, such as mannose residues, may also be important for sorting and transport of lysosomal enzymes (see Gabel *et al.* 1984; Stahl *et al.* 1984).

Morphological studies have also suggested that some autophagic vacuoles may receive lysosomal enzymes directly from the smooth endoplasmic reticulum which is believed to take part in the segregation of cytoplasmic components into autophagic vacuoles (see Ericsson 1969). Acid phosphatase-positive smooth endoplasmic reticulum has been shown to occur at the inner "trans" aspect of the Golgi apparatus. This specialized part of the endoplasmic reticulum (ER) has been named GERL because of its association with the Golgi apparatus (G) and since it appears to produce various types of lysosomes (L) (see Novikoff 1976).

Lysosomes in skeletal muscle

Cytoplasmic particles containing a representative number of latent acid hydrolases have been isolated from skeletal muscle suggesting the presence of lysosomes in this tissue (see Bird 1975). Zonal centrifugation studies have also indicated that the main part of lysosomal enzymes, such as acid phosphatase and cathepsin D, derives from muscle fibres rather than from macrophages or other connective tissue cells (Canonica and Bird 1970).

Examples of morphologically demonstrated lysosomes in normal skeletal muscle are rare in the literature. Under conditions of physiological or pathological stress they have, however, been clearly demonstrated and they have been suggested to participate in intracellular digestive processes (see Bird 1975). The mechanism of formation of muscle fibre lysosomes is not entirely clear but the membrane of such lysosomes has been suggested to derive from various sources such as the t-system, sarcoplasmic reticulum and Golgi apparatus (see Bird 1975).

AIM OF THE PRESENT STUDY

The aim of the present study was to further characterize the endocytic activity of skeletal muscle and its relationship to the lysosome system of muscle fibres. This has been done by the use of biochemical, radiochemical, histochemical and ultrastructural methods. Some of the results have also been evaluated in parallel with electrophysiological studies.

Particular attention has been paid to the nervous influence over endocytic activity and the lysosome system of muscle and the results are discussed mainly in terms of trophic influences between nerve and muscle.

METHODOLOGICAL CONSIDERATIONS

"'Tis a lesson you should heed,

Try, try again.

If at first you don't succeed,

Try, try again."

(W. E. Hickson: Try and Try Again)

GENERAL

Animals and muscles

Mice were chosen as the experimental animals for this study since most of the previous work on endocytosis in skeletal muscle has been carried out on this species. The size of muscles such as the extensor digitorum longus (EDL) of mice is suitable for *in vitro* studies in which large muscles can cause difficulties due to diffusion restrictions. The size of mice is also advantageous for *in vivo* studies requiring injections of large doses of extracellular markers such as HRP. The experiments were performed on adult male NMRI mice weighing about 30g.

Muscles used for the different studies were diaphragm, tibialis anterior, EDL and soleus muscles. Denervation of the muscles was performed under diethyl-ether anaesthesia. The left hemidiaphragm was denervated by sectioning the left phrenic nerve where it passes from the heart to the diaphragm. Tibialis anterior and EDL muscles were denervated by sectioning the deep peroneal nerve just above the knee. For studies on the effects of reinnervation, this nerve was crushed in the same position using a pair of fine-tipped forceps. The soleus muscle was denervated by sectioning the tibial nerve in fossa poplitea.

Botulinum toxin-induced muscle paralysis (paper VII) was produced by injecting crystalline *Clostridium botulinum* toxin type A (haemagglutinin + neurotoxin, M.W. ~ 150,000) into the anterolateral region of the right hind leg under diethyl-ether anaesthesia. An amount of the toxin approximately equal to two times the mouse LD₅₀ after intraperitoneal injection, dissolved in 0.05 ml of a gelatine-phosphate buffer, pH 6.6 (Ambache 1949), was injected in a single bolus. This dosage has previously been shown to produce complete paralysis of the EDL muscle as judged from indirect stimulation *in vitro* (Mathers and Thesleff 1978). To ensure that muscle paralysis occurred, the inability of the animals to spread the toes of the injected leg was checked before sacrifice.

***In vivo* uptake of horseradish peroxidase**

For *in vivo* studies of the uptake of HRP, 20 mg of HRP (Sigma, Type II) dissolved in 0.2 ml 0.9% saline was injected into a tail vein at different times (15 min-48 hr) before the animal was sacrificed. Control animals received an equal volume of saline only. EDL and tibialis anterior muscles to be used for biochemical studies and diaphragms to be used for biochemical or gross morphological studies were dissected and then rinsed overnight in four changes (100-150 ml each) of a continuously oxygenated Ringer's solution at 4°C in order to reduce extracellular HRP. Muscles to be used for histochemical or ultrastructural studies were fixed and processed as described below.

***In vitro* uptake of horseradish peroxidase and ³H-inulin**

In vitro uptake studies were performed only on EDL and soleus muscles. The small size of these muscles minimizes diffusion restrictions and both muscles can be dissected with intact tendons. Following incubation in the presence of ³H-inulin and/or HRP at 37°C the muscles were rinsed 4x1 hr or overnight in four changes (100-150 ml each) of continuously oxygenated Ringer's solution at 4°C. Studies on the labelling of the extracellular space were carried out by incubating at about 0-4°C (using an ice-water mixture for cooling). These muscles were not rinsed following incubation but were briefly blotted on filter paper before freezing as described below.

Homogenization of muscles

Following incubation or rinsing, muscles from *in vivo* or *in vitro* uptake studies were blotted dry, weighed, rapidly frozen on a glass dish cooled on dry ice and stored individually in sealed plastic tubes at -20°C. Before blotting, diaphragms were cut into strips considered to contain a concentration of endplates and strips considered to be devoid of endplates. The frozen muscles were later homogenized in 2 ml (EDL, soleus, diaphragm strips) or 6 ml (tibialis anterior) of ice-cold 0.3 M sucrose using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). During the homogenization, the homogenate was continuously cooled in an ice-water mixture. The resulting homogenate contained approximately 0.5-1.5% muscle tissue (wet weight).

BIOCHEMICAL AND RADIOCHEMICAL STUDIES

Details of the various assays used are given in the original papers and will only be briefly summarized here.

Determination of ^3H -inulin uptake

The amount of tritium retained in EDL muscles after incubation in the presence of ^3H -inulin was determined by liquid scintillation counting. In paper IV the radioactivity in each incubation solution was also determined and this information was used for converting muscle radioactivity content into μl extracellular fluid equivalents.

Counting efficiency was determined from standards prepared with ^3H -toluene of known specific activity and carbon tetrachloride as quenching agent.

Horseradish peroxidase assay

After appropriate dilution of the muscle homogenates the peroxidase activity was assayed according to the method of Lundquist and Josefsson (1971) using a 0.1 M maleic acid-NaOH buffer, pH 6.0. This method is very sensitive with a detection limit of about 0.2 ng HRP (approximately 5×10^{-15} moles). In the original report (Lundquist and Josefsson 1971) a stable liquid preparation of glucose oxidase was used. In the present work a lyophilized glucose oxidase preparation (Boehringer, Grade I) was used. A concentration of 1 $\mu\text{g}/\text{ml}$ in the reagent solution was found to give optimal sensitivity to the peroxidase assay.

Assays of peroxidase activity were always run in parallel with standards of known HRP concentrations. In paper IV the peroxidase activity in each incubation solution was also determined and this information was used for converting muscle HRP content into μl extracellular fluid equivalents.

The catalase activity of muscle tissue is too low to influence the measurement of endogenous peroxidase activity in normal muscle when a homogenate containing 1% muscle tissue (wet weight) is used and the incubation is carried out at pH 7.0 (Lundquist and Josefsson 1971). The specific activity of catalase in muscle has been shown to increase by about 100-220% after denervation or chloroquine treatment (Stauber *et al.* 1977). Even at these higher activities dilution of the homogenate and the low pH (6.0) used in the peroxidase assay negate any influence of catalase.

Assays of lysosomal enzymes

Acid phosphatase activity in the muscle homogenates was measured with 4-methylumbelliferyl phosphate as substrate. This substrate was first used by Robinson and Willcox (1969) for measuring acid phosphatase activity. The method has been adapted for skeletal muscle homogenates by Lundquist (personal communication).

N-acetyl- β -D-glucosaminidase activity was measured with 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide as substrate. This substrate was first used by Leaback and Walker (1961) for measuring N-acetyl- β -D-glucosaminidase activity. The method as used in the present work was adapted for skeletal muscle homogenates by Lundquist (personal communication).

Cathepsin D activity was measured with haemoglobin as substrate essentially as described by Barret and Heath (1977).

Protein assay

The amount of protein in the homogenates was determined, after appropriate dilution, by the method of Lowry *et al.* (1951).

MORPHOLOGICAL STUDIES

Detailed descriptions of the different solutions and procedures used are given in the original papers and will only be briefly summarized here.

Fixation of muscles

Tibialis anterior muscles to be used for histochemical studies were fixed using a buffered formaldehyde fixative at pH 7.2. To ensure that the whole muscles were properly fixed, cold fixative was injected with a 0.4 mm hypodermic needle into the muscles followed by immersion in cold fixative overnight.

For ultrastructural studies a fixative containing 2% glutaraldehyde and 1% formaldehyde (freshly prepared from paraformaldehyde as described by Robertson *et al.* 1963) in 0.12 M phosphate buffer, pH 7.3, was used. Tibialis anterior muscles to be used for ultrastructural studies were fixed by perfusion of the whole animal with fixative at room temperature, by injection of a small volume of cold fixative into the muscles using a 0.4 mm hypodermic needle, and by immersion in cold fixative for an additional 2 hr at 4°C.

Preparations for ultrastructural studies were, after fixation, rinsing and staining for HRP (see below), post-fixed in osmium tetroxide.

Histochemical staining of acid phosphatase

Histochemical staining of acid phosphatase was performed as described by Barka and Anderson (1962) using naphthol AS-BI phosphate (phosphate ester of 6-bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide) as substrate and hexazonium pararosaniline as a simultaneous coupling agent. Control sections were pre-treated with 10 mM sodium fluoride (NaF), an inhibitor of acid phosphatase (Reiner *et al.* 1955; Neil and Horner 1964), or were incubated in the presence of NaF.

Staining of horseradish peroxidase

All staining of HRP was carried out using 3,3'-diaminobenzidine as substrate. This substrate was suggested by Graham and Karnovsky (1966) to be superior to benzidine and o-dianisidine for ultrastructural cytochemistry because of the greater electron opacity of its reaction product.

Histochemical staining

Histochemical staining of HRP was carried out essentially as described by Graham and Karnovsky (1966).

Cytochemical staining for ultrastructural studies

In a previous ultrastructural study of HRP uptake in denervated skeletal muscle (Libelius *et al.* 1979a) it was observed that a stronger reaction product was obtained if hydrogen peroxide was continuously generated by glucose oxidase in the staining medium rather than added directly. In the present work such a glucose oxidase staining technique was therefore used.

Most of the ultrastructural studies were performed on single muscle fibres or small bundles of fibres which were carefully dissected from fixed tibialis anterior muscles using fine-tipped forceps or thin hypodermic needles. The tibialis anterior muscle was chosen for these studies because the deep part of this muscle contains less connective tissue than is usually found surrounding muscles. This relative lack of connective tissue facilitates the dissection of single fibres from the muscle. Dissection and all subsequent handling of the fibres was carried out under a dissecting microscope.

The method for selecting single fibres with high segmental peroxidase staining was developed because histochemical studies (paper II) had shown that such segments are non-randomly distributed in denervated muscles and because blocks of muscle tissue or whole tissues give

uneven staining for peroxidase in the centre of the blocks or tissues (Libelius *et al.* 1979a; paper III). Using the single fibre method a good yield of material, homogeneously stained and embedded, was obtained for ultrastructural study.

Gross morphological staining of whole muscles

In paper III whole diaphragms were stained for HRP in order to determine the localization of segments with high HRP content in denervated muscles. To achieve a good preparation which clearly showed the localization of the segments it was necessary to develop staining conditions which give a minimum of background staining. The background staining was found to be less in muscles fixed with the formaldehyde fixative used for histochemical studies than in muscles fixed with the glutaraldehyde-formaldehyde mixture used for ultrastructural studies. The background staining was further reduced by using low concentrations of 3,3'-diaminobenzidine and glucose oxidase in the staining medium.

RESULTS AND DISCUSSION

BIOCHEMICAL AND RADIOCHEMICAL UPTAKE STUDIES

^3H -inulin uptake (papers I and IV)

The labelling of the extracellular space with ^3H -inulin (paper IV) was studied by incubating EDL muscles at about $0-4^\circ\text{C}$ (cooled by an ice-water mixture), a temperature at which no intracellular uptake is assumed to occur. The extracellular space equilibrated with the marker within 30-60 min of incubation. The size of the space labelled was about $1.750\ \mu\text{l}/\text{mg}$ muscle protein in innervated muscles and $1.945\ \mu\text{l}/\text{mg}$ muscle protein in 6-day denervated muscles. In both cases this represents about 21% of the muscle weights (μl extracellular volume/mg muscle wet weight). Washing the muscles for 4×1 hr or overnight in four changes of Ringer's solution at 4°C reduced the ^3H -inulin content 97-98%.

The intracellular uptake of ^3H -inulin at 37°C in innervated and 6-day denervated EDL muscles was shown by Libelius *et al.* (1978a) to obey bulk kinetics, *i.e.* a large excess of unlabelled inulin in the incubation solution did not affect the uptake of ^3H -inulin. In paper IV the intracellular accumulation of ^3H -inulin was shown to proceed linearly for at least four hours of incubation. The rates of intracellular uptake were $0.086\ \mu\text{l}$ extracellular fluid/mg muscle protein/hr in innervated muscles and $0.152\ \mu\text{l}$ extracellular fluid/mg muscle protein/hr in denervated muscles, which represents 4.9% and 7.8% of the apparent extracellular volume/hr, respectively.

It should be noted that a relatively short period of incubation, say two hours, with ^3H -inulin results in an intracellular accumulation in innervated muscles which represents only about 9.8% of the ^3H -inulin present in the extracellular space. If 3% of the extracellular ^3H -inulin remains after overnight washing this will represent about 23% of the final uptake value. Rather long incubation periods may therefore be advisable when ^3H -inulin is used as the uptake marker. In paper I incubation periods of four hours were used. In this situation the contribution of extracellular ^3H -inulin should be no more than 10-15% of the presented uptake values for innervated and denervated muscles.

It should be noted that the endocytic activity of the muscles as measured above is large enough to turn over the entire extracellular volume of the muscles in just over 20 hours in innervated muscles and in less than 13 hours in denervated muscles. Considering also the large mass of skeletal muscle in the body this raises interesting possibilities concerning the role of muscle tissue for the clearance and/or turnover of various extracellular substances.

Horseradish peroxidase uptake (paper IV)

The extracellular space was found to equilibrate with HRP at a rate similar to that seen with ^3H -inulin. The space labelled was about 1.160 $\mu\text{l}/\text{mg}$ muscle protein in innervated muscles and 1.265 $\mu\text{l}/\text{mg}$ muscle protein in 6-day denervated muscles. Both these values represent about 14% of the muscle weights, a value which is less than that measured with ^3H -inulin. Washing the muscles for 4x1 hr or overnight in four changes of Ringer's solution reduced the extracellular HRP content more than 95%. The reason why the apparent extracellular space labelled with HRP is smaller than that labelled with ^3H -inulin is not clear.

The uptake of HRP (when used at 82.7 $\mu\text{g}/\text{ml}$ in the incubation solution) at 37°C proceeds at a rate which is 10-20 times higher than that observed for ^3H -inulin uptake. This observation prompted a closer investigation of the characteristics of the HRP uptake in innervated and denervated skeletal muscles.

Adsorptive uptake

In contrast to ^3H -inulin uptake the uptake of HRP at 37°C was found to obey saturation kinetics, *i.e.* the uptake did not increase linearly but levelled off as the concentration of HRP in the incubation solution was increased. This suggested that some kind of adsorptive endocytic mechanism was operating. To account for this it was considered possible that the HRP used (Sigma, Type II) might become unspecifically adsorbed onto the muscle fibre membranes since this HRP preparation, although not pure, contains mainly basic isoenzymes which at physiological pH could become attracted to negative charges on cell membranes. This possibility was, however, excluded since purified acidic and basic isoenzymes (Sigma, Types VIII and IX) were taken up at similar rates by innervated and denervated muscles and because the extracellular space labelled with HRP was smaller than that labelled with ^3H -inulin. This is the opposite to what would be expected if HRP was adsorbed onto cell membranes to any large extent.

A second possibility considered was that after uptake HRP might become adsorbed onto intracellular membranes thereby being retained in the muscle fibres more strongly than ^3H -inulin. Measurements of the disappearance of the two markers from the muscles, however, showed that they disappear with similar rates.

Receptor-mediated endocytosis via mannose recognition

In the literature some reports had suggested that HRP in some cases was specifically taken up or bound to certain cell types because of its content of mannose residues. The rapid clearance of HRP following infusion into the rat was shown to be mediated by specific recognition sites and was inhibited in the presence of the mannose polymer mannan from yeast (Rodman *et al.* 1978).

In rabbit alveolar macrophages HRP was also shown to be taken up by a mechanism of adsorptive pinocytosis (Kaplan and Nielsen 1978), and in rat alveolar macrophages HRP was shown to compete with the receptor-mediated uptake of various glycoproteins and glycoconjugates with terminal mannose, N-acetylglucosamin or glucose (Stahl *et al.* 1978). HRP had also been found to bind to mannose-specific receptors on various cell types in fixed, frozen sections from different rat tissues (Straus 1981; 1983a; 1983b).

The above observations raised the possibility that the adsorptive uptake of HRP in skeletal muscle also depended on the binding of HRP to mannose-specific receptors. In accordance with this, yeast mannan was shown to be a potent blocker of the HRP uptake in both innervated and denervated EDL muscles. At a concentration of 1 mg/ml, yeast mannan caused more than a 90% reduction in the HRP uptake (muscles incubated in the presence of 10 µg/ml of HRP). In the same innervated muscles this concentration of yeast mannan caused only about a 15% reduction of the ³H-inulin uptake (unpublished observations). Mannose was also found to be a more potent blocker of the HRP uptake than other sugars or sugar derivatives such as sucrose and mannose-6-phosphate. Another glycoprotein which is also relatively rich in mannose residues, ribonuclease B, was also shown to inhibit HRP uptake whereas the unglycosylated protein ribonuclease A showed no such inhibitory activity.

Yeast mannan and ribonuclease B were much more potent inhibitors of HRP uptake than was mannose itself, suggesting that the binding of HRP and the inhibitors involves recognition of sequences of mannose, and possibly combinations with other sugar residues, rather than single mannose residues. This is in agreement with results reported for endocytosis of mannose-containing glycopeptides and glycoproteins by isolated rat hepatic reticuloendothelial cells (Maynard and Baenziger 1981) for which a minimum required oligosaccharide structure could be determined. Similarly, rabbit alveolar macrophages have been shown to take up glycoconjugates with a high mannose content at much higher rates than glycoconjugates with a lower mannose content (Hoppe and Lee 1983). Alternatively, the large ligands may act as multivalent ligands which can interact with multiple receptors. This possibility has also been suggested for the binding of lysosomal enzymes to the phosphomannosyl recognition system (see Sly and Fischer 1982).

Influence of divalent cations and pH on horseradish peroxidase uptake

Also in accordance with a receptor-mediated uptake of HRP in skeletal muscle were the observations that removal of divalent cations or lowering the pH of the incubation solution to 6.4 reduces the uptake. Similar effects have been observed for the receptor-mediated uptake of a number of other ligands in different cell types (see Kaplan 1981; Anderson and Kaplan 1983; Brown *et al.* 1983; Steinman *et al.* 1983; Shepherd and Stahl 1984). The low pH or low concentrations of divalent cations reduces the affinity of receptors for ligand, thereby reducing the uptake. Physiologically these mechanisms may be of importance for releasing the ligand from the

receptors in acidic intracellular compartments (endosomes, receptosomes and/or lysosomes) after uptake (see Kaplan 1981; Brown *et al.* 1983; Steinman *et al.* 1983; Shepherd and Stahl 1984; Maxfield 1985). Following release of ligand the receptors are believed to return to the cell surface for reutilization, whereas the ligand remains trapped inside the cells.

Drugs influencing horseradish peroxidase uptake

Weak bases such as chloroquine have been suggested to inhibit receptor-mediated endocytosis by increasing the pH of the intracellular organelles where uncoupling of ligand from receptor normally occurs, thereby inhibiting receptor recycling and intracellular delivery of ligand (see Brown *et al.* 1983; Shepherd *et al.* 1983; Steinman *et al.* 1983; Shepherd and Stahl 1984; Maxfield 1985). In cultures of embryonic skeletal muscle tissue, chloroquine has also been suggested to inhibit the fusion of lysosomes with coated vesicles (Libby *et al.* 1980; Bursztajn and Libby 1981). In the present study (paper IV) chloroquine was also found to inhibit the *in vitro* uptake of HRP in innervated and denervated muscles. Another drug which inhibited HRP uptake was trifluoperazine, a drug which may act by inhibiting the calcium-regulatory protein calmodulin, which is also believed to be important for receptor-mediated internalization of ligands (Salisbury *et al.* 1980; 1981; 1983; Hebbert and Morgan 1985). Monodansylcadaverine was also shown to inhibit the uptake of HRP and this drug has been suggested to inhibit receptor-mediated endocytosis by inhibition of the enzyme transglutaminase. The inhibition of transglutaminase was suggested to result in inhibition of protein cross-linking and thereby inhibition of the clustering of ligand-receptor complexes in coated pits (Davies *et al.* 1980). Other mechanisms, however, may also be of importance (see Anderson and Kaplan 1983; Salisbury *et al.* 1983; Davies and Murtaugh 1984; Davies *et al.* 1984).

Conclusions

The data presented in paper IV suggest that there exist, on the membranes of skeletal muscle fibres, receptors which recognize and mediate the internalization of macromolecules rich in mannose residues. The physiological and/or pathophysiological role of these receptors remains obscure. Some possible functions will be discussed below under GENERAL DISCUSSION.

Endocytosis after denervation, botulinum toxin poisoning and during reinnervation (papers I, II, IV and VII)

The *in vivo* or *in vitro* uptake of the extracellular markers ^3H -inulin and HRP by skeletal muscle increases significantly 2-4 days after nerve section (Libelius *et al.* 1978a; 1979a; papers I and II).

The uptake then remains at a high level for prolonged periods of time after denervation. The longest time so far studied is seven weeks after denervation (table I). In all cases the increase in uptake after denervation is evident whether expressed relative to muscle weight, muscle protein content or per muscle (papers I and II; table I). The increase in uptake after denervation thus represents a true increase in the rate of endocytic activity of the muscles.

Table I Uptake of horseradish peroxidase (HRP) in innervated and 7 weeks denervated extensor digitorum longus (EDL) muscles

Muscle	Specific uptake	Total uptake
	(ng HRP/mg muscle protein)	(ng HRP/muscle)
	Mean $\pm$ SE, n=5	Mean $\pm$ SE, n=5
Innervated EDL	106.8 $\pm$ 4.7	190.9 $\pm$ 5.2
Denervated EDL	369.5 $\pm$ 34.7	350.3 $\pm$ 21.8

Muscles were incubated in the presence of HRP (100 μ g/ml) for 2.5 hr at 37°C and then rinsed overnight in four changes of Ringer's solution at 4°C. Values given have not been corrected for endogenous peroxidase activity.

The increase in uptake after denervation is much larger for HRP than for ^3H -inulin (paper IV). When HRP was used at a concentration of 82.7 μ g/ml in the incubation solution the rate of uptake of HRP was approximately 8 times as high as the uptake of ^3H -inulin in the same innervated muscles whereas in denervated muscles the HRP uptake was approximately 19 times as high as the ^3H -inulin uptake. The reason for this difference is not clear. Possible explanations include denervation induced changes in the affinity and/or number of mannose binding receptors present on the cell surface and/or changes in the rate at which these receptors are being reutilized.

If muscles are allowed to become reinnervated, the uptake of extracellular markers begins to decline at around the same time as signs of innervation (presence of spontaneous miniature endplate potentials and muscle twitch after nerve stimulation) start to appear (9 days after nerve crush, paper I). Three weeks after nerve crush the uptake activity is the same as in innervated control muscles.

Many denervation changes in skeletal muscle, such as the appearance of extrajunctional acetylcholine receptors and the development of tetrodotoxin resistant action potentials, have been suggested to be triggered by the presence of degenerating axons and nerve terminals in the muscle (Vrbová 1967; Cangiano *et al.* 1984; Cangiano 1985). To test if the presence of nerve degradation products is necessary for the increase in endocytic activity of muscles after denervation, muscle paralysis was produced by a single local injection of botulinum toxin into the leg. This neurotoxin inhibits the evoked release of acetylcholine at the neuromuscular junction (Burgen *et al.* 1949) without causing any degenerative changes in motor nerve terminals (Thesleff 1960; Duchon 1971). The *in vitro* uptake of HRP was studied in EDL muscles 6 and 11 days after toxin injection (paper VII). Six days after poisoning there was no increase in the uptake of HRP. This was

confirmed in a separate study on the *in vivo* uptake of HRP in EDL muscles 7 days after toxin injection (unpublished observations). Eleven days after toxin injection, however, the *in vitro* uptake of HRP, expressed per g muscle protein, was significantly higher than that of buffer-treated control muscles. At this time after injection the uptake still did not reach the size of that in denervated muscles. If the uptake was expressed per muscle, the increase in botulinum poisoned muscles, compared to buffer treated control muscles, did not reach statistical significance ($0.05 < p < 0.10$) but the uptake in botulinum toxin poisoned muscles was significantly lower than that in denervated muscles ($p < 0.02$). For actual values see paper VII.

Despite the differences observed between denervated and botulinum toxin-poisoned muscles the conclusion is that botulinum toxin-induced muscle paralysis and denervation cause qualitatively similar changes in endocytic activity of muscles. This conclusion receives additional support from the fact that histochemical studies of 11-day botulinum toxin-poisoned muscles show the presence of segments with high peroxidase content in muscle fibres (paper VII) similar to what is seen in denervated muscles (paper II). These results indicate that increased endocytic activity may occur in skeletal muscle without the presence of degenerating axons.

The reason for the smaller and slower increase in HRP uptake after botulinum toxin paralysis compared to after denervation is not clear. Increases in other denervation-like changes, such as the number of extrajunctional acetylcholine receptors and the appearance of tetrodotoxin-resistant action potentials, have also been observed to be less after botulinum toxin-induced muscle paralysis than after surgical denervation (Pestronk *et al.* 1976; Mathers and Thesleff 1978). Botulinum toxin prevents the evoked release of acetylcholine (Burgess *et al.* 1949), the spontaneous release of normal "fast" miniature endplate potentials (Brooks 1956) and reduces the molecular release of acetylcholine from nerve terminals (Polak *et al.* 1981; Doležal *et al.* 1983). Some molecular leakage remains, however, and a "new" type of spontaneous endplate potentials of slower time course appears 6-10 days after poisoning (see Thesleff and Molgó 1983; Kim *et al.* 1984). This remaining acetylcholine influence on the muscle may be responsible for preventing full scale denervation effects but other possibilities, such as continued release of some neurotrophic factor or the absence of nerve degradation products, cannot be excluded.

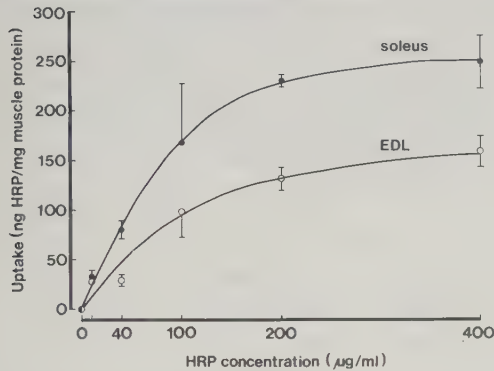
Uptake in different muscles

Innervated muscles

Normal innervated muscles show differences in the amounts of accumulated marker after *in vivo* or *in vitro* incubation with markers such as ^3H -inulin or HRP. Libelius *et al.* (1978a) noted that the uptake of ^3H -inulin per mg muscle wet weight after *in vitro* incubation was higher in soleus muscles than in EDL muscles. The same was found for the *in vivo* uptake of HRP in these muscles (Libelius *et al.* 1979a). Fig. 1 also shows that the uptake of HRP per mg muscle protein

after *in vitro* incubation is higher in soleus than in EDL muscles. It may thus be concluded that the uptake of extracellular markers, expressed per unit muscle wet weight or per unit muscle protein, is higher in innervated muscles composed largely of "red" (type I) muscle fibres (soleus) than in innervated muscles containing mainly "white" (type II) muscle fibres (EDL) (for fibre type composition of these muscles see Schmalbruch 1985).

Fig.1 Uptake of horseradish peroxidase (HRP) in innervated extensor digitorum longus (EDL) (O) and soleus (●) muscles during 2.5 hr incubation at 37°C in the presence of different horseradish peroxidase concentrations



Following incubation the muscles were rinsed 4x1 hr in oxygenated Ringer's solution at 4°C. Endogenous peroxidase activity (equivalent to 4.2 and 16.8 ng HRP/mg muscle protein for EDL and soleus muscles respectively) has been subtracted from the values shown. Mean values $\pm$ SE (n=3 for all points).

Denervated muscles (papers III and VI)

In all muscles studied, the uptake of extracellular markers has been shown to increase after denervation. The size of the increase, however, varies between different muscles. Libelius *et al.* (1978a) observed that the *in vitro* increase in ^3H -inulin uptake per mg muscle wet weight as a result of denervation was more pronounced in soleus than in EDL muscles. A similar result was obtained for the *in vivo* uptake of HRP in these muscles after denervation (Libelius *et al.* 1979a). Table II also shows that the denervation-induced increase in the *in vitro* uptake of HRP per mg muscle protein is larger in soleus muscles than in EDL muscles. In paper VI it was observed that the *in vivo* increase in HRP uptake per g muscle protein after denervation is more pronounced in EDL than in tibialis anterior muscles.

Table II Uptake of horseradish peroxidase (HRP) in innervated and 21-day denervated extensor digitorum longus (EDL) and soleus muscles

Muscle	Specific uptake (ng HRP/mg muscle protein)	Total uptake (ng HRP/muscle)
	Mean $\pm$ SE, n=5	Mean $\pm$ SE, n=5
Innervated EDL	110.2 $\pm$ 18.4	206.4 $\pm$ 29.0
Denervated EDL	287.9 $\pm$ 35.8	365.8 $\pm$ 46.5
Innervated soleus	162.7 $\pm$ 9.0	176.8 $\pm$ 9.7
Denervated soleus	544.3 $\pm$ 36.6	252.5 $\pm$ 29.6

Muscles were incubated in the presence of HRP (100 μ g/ml) for 2.5 hr at 37°C and then rinsed overnight in four changes of Ringer's solution at 4°C. Values given have not been corrected for endogenous peroxidase activity.

Expressing the increase in uptake after denervation in relation to muscle wet weight or muscle protein content may be misleading since different muscles may show different degrees of atrophy. In paper III it was also shown that the increase in uptake after denervation occurs primarily in the endplate region of muscle fibres. If this increase in uptake is of the same absolute magnitude in fibres of different muscles but the fibres vary in protein content due to differences in *e.g.* the length of the muscle fibres, then the increase in uptake relative to muscle protein content will be smaller in muscles containing longer muscle fibres.

Another way of expressing the increase in uptake after denervation is to look at the total increase in uptake of whole muscles. Expressed in this way the increase in uptake after denervation is larger in tibialis anterior than in EDL muscles and smallest in soleus muscles. This is as expected since the number of muscle fibres is largest in tibialis anterior and smallest in soleus muscles (for fibre numbers in rat muscles see Schmalbruch 1985).

Whichever way is chosen for expressing the uptake it seems clear that different muscles, composed of different proportions of fibre types ("red", "white" and "intermediate"), react in a qualitatively similar way by increasing their uptake of extracellular markers after denervation. In the histochemical study, which was performed on tibialis anterior muscles (see below), no attempts were made, however, to confirm that the increase in uptake after denervation occurs in both "red" and "white" muscle fibres.

Effects of chloroquine on horseradish peroxidase uptake *in vivo* (paper VI)

In previous studies it was shown that in some muscle disorders, such as denervation and muscular dystrophies in animals, increased endocytosis accompanies or precedes increases in

lysosomal enzyme activities and degeneration of muscle fibres (Libelius *et al.* 1978a; 1978b; 1979a; 1979b; 1981). An *in vitro* model was also developed which showed that increased endocytosis, lysosomal activation and myofibrillar degeneration with vacuolation can be triggered by incubating muscles in the presence of the polycationic protein protamine (Jirmanová *et al.* 1977; Libelius and Lundquist 1978). On the basis of these studies a working hypothesis was proposed in which increased endocytosis may initiate lysosomal activation and muscle degeneration (see Libelius *et al.* 1978c; Thesleff *et al.* 1979; Thesleff and Libelius 1983).

In an attempt to expand these studies to other pathological muscle conditions chloroquine diphosphate (50 mg/kg twice daily) was administered intraperitoneally to a number of mice for 22 days and the effects on the *in vivo* uptake of HRP was studied in innervated and 14-day denervated EDL and tibialis anterior muscles. Chloroquine has been shown to cause a severe myopathy as a side effect in humans (Whisnant *et al.* 1963) and has also been shown to cause histological and ultrastructural changes in skeletal muscles of experimental animals such as rats, rabbits and chickens (Nelson and Fitzhugh 1948; Smith and O'Grady 1966; MacDonald and Engel 1970; Aguayo and Hudgson 1970; Klinghardt 1974; Drenckhahn and Lüllmann-Rauch 1979; Schmalbruch 1980; Stauber *et al.* 1981a; 1981b; Trout *et al.* 1981b; 1981c; Trout *et al.* 1982).

The dose of chloroquine used in the present work was considered a high dose. Giving the total daily dose in a single injection killed the animals. When the daily dose was divided into two injections, however, it was well tolerated. Throughout the experimental study the animals moved freely in their cages without any obvious signs of impaired muscle function. There was no reduction in the weights of chloroquine-treated animals as compared to control animals injected with saline only. Muscle weights did not differ significantly between muscles from chloroquine-treated animals and muscles from saline treated controls although there was a slight tendency towards a reduction in the weights of denervated tibialis anterior muscles from chloroquine-treated animals. Similarly, the protein content of muscles showed only small changes after chloroquine treatment. A decrease of about 10% occurred in denervated muscles from chloroquine-treated animals but reached statistical significance ($p < 0.05$) only in denervated tibialis anterior muscles. These observations suggest that mice may be relatively resistant to the effects of chloroquine. Lower daily doses of the drug have been shown to produce both weight loss and signs of muscle weakness in rats (MacDonald and Engel 1970; Schmalbruch 1980).

The chloroquine treatment did not cause any changes in the *in vivo* uptake of HRP in any of the muscles studied (EDL and tibialis anterior). The EDL muscle is a "white" muscle and the tibialis anterior muscle is composed of a mixture of fibre types. Such muscles are known to be less sensitive to the effects of chloroquine than are "red" muscles (Smith and O'Grady 1966; MacDonald and Engel 1970; Schmalbruch 1980; Trout *et al.* 1981c). However, other observations on muscle lysosomal enzyme activities and ultrastructure (discussed below), clearly showed that the muscles had been affected by the chloroquine treatment and lack of effect on the muscles can therefore not have been the reason for the absence of effects on the HRP uptake. In order to study the HRP uptake in muscles affected more severely by chloroquine, a predominantly

"red" muscle may be chosen. Such uptake studies may, however, be complicated by the fact that chloroquine causes necrosis in "red" muscle fibres (fibres rich in mitochondria) (Schmalbruch 1980) and the infiltration of inflammatory cells may then contribute to the uptake of extracellular markers when measured in whole muscles.

The results obtained in this study cannot be directly incorporated into the working hypothesis that increased endocytosis causes lysosomal activation and muscle fibre degeneration since no effect was observed on endocytic activity. The study shows, however, that alterations in the lysosomal system of skeletal muscle may occur without any changes in endocytic activity.

From the effects of chloroquine on the *in vitro* uptake of HRP (discussed above) it could have been expected that this drug would inhibit HRP uptake also *in vivo*. The most likely reason why this did not occur is probably that high enough concentrations of the drug were not obtained *in vivo*.

LYSOSOMAL ENZYME ACTIVITIES

The activities of the different lysosomal enzymes studied did not always change in the same way under the different experimental conditions used. The different enzymes will therefore first be discussed separately followed by a discussion of more general matters.

Acid phosphatase (papers II, III and VI)

Acid phosphatase was the first lysosomal enzyme studied (see de Duve 1969) and is probably still one of the most studied lysosomal enzymes. In skeletal muscle the activity of this enzyme has been shown to increase after denervation (see Weinstock and Iodice 1969).

The activity of acid phosphatase has been measured biochemically by the use of a variety of different phosphate esters as substrates. The hydrolysis of such phosphate esters at acidic conditions may, however, also be catalyzed by enzymes which are not believed to be of lysosomal origin (see Beaufay 1972; Dean 1975; Barret and Heath 1977).

The substrates used in the present work were 4-methylumbelliferyl phosphate for biochemistry and naphthol AS-BI phosphate for histochemistry. Using these substrates the activities of different acid phosphatase isoenzymes, isolated from gastrocnemius muscles of rabbits, were studied by Meijer *et al.* (1979). Three distinct isoenzymes were separated by gel filtration and polyacrylamide gel electrophoresis and all three isoenzymes were found to split 4-methylumbelliferyl phosphate whereas only two of the isoenzymes were active against naphthol AS-BI phosphate. Only the two isoenzymes which split both substrates were increased in muscles from vitamin E deficient rabbits. Based on this observation and molecular weight considerations it was considered likely that the two isoenzymes which split both substrates were of lysosomal origin whereas that which split only 4-methylumbelliferyl phosphate was most likely not lysosomal. The

contribution of non-lysosomal acid phosphatase to the total activity measured with 4-methylumbelliferyl phosphate as substrate was also suggested to explain the observation that in vitamin E deficient rabbits the specific activity of acid phosphatase was 17 times greater than in control muscles when naphthol AS-BI phosphate was used as substrate, but only two times greater when 4-methylumbelliferyl phosphate was used (Meijer *et al.* 1979).

In the present work the specific activity of acid phosphatase was found to increase after denervation (papers II, III and VI), and histochemical studies (paper II) showed increased acid phosphatase staining in spindle-shaped segments of denervated muscle fibres (see below). In paper II it was noted, however, that in denervated tibialis anterior muscles there was no apparent increase in the acid phosphatase activity if this was expressed as total activity per muscle rather than as activity per g muscle protein. This indicated that if this enzyme was spared from the overall protein loss occurring in denervated muscles then the increase in specific activity could merely be a result of the decrease in protein content of the denervated muscles. The increase in acid phosphatase staining shown to occur in segments of the denervated muscle fibres would then have to be the result of a redistribution within the muscle of pre-existing enzyme. The studies of Meijer *et al.* (1979) referred to above, however, suggest the possibility that the unchanged acid phosphatase activity per muscle after denervation may be the result of an increase in total lysosomal acid phosphatase activity per muscle and a concomitant decrease in total activity per muscle of the non-lysosomal acid phosphatase. The results obtained in paper VI support this possibility. The results on the specific activity of acid phosphatase in innervated and 14-day denervated EDL and tibialis anterior muscles reported in paper VI are reproduced in table III together with the corresponding values for total activity per muscle.

Table III Acid phosphatase activity in innervated (Inn) and 14-day denervated (Den) tibialis anterior (tib ant) and extensor digitorum longus (EDL) muscles

Muscle	Specific activity (μmol 4-methylumbelliferone liberated/min/g muscle protein)	Total activity (μmol 4-methylumbelliferone liberated/min/muscle)
	Mean $\pm$ SE, n=12	Mean $\pm$ SE, n=12
Inn tib ant	5.2 $\pm$ 0.1	54.2 $\pm$ 1.5
Den tib ant	7.8 $\pm$ 0.3	48.6 $\pm$ 2.5
Inn EDL	4.7 $\pm$ 0.1	8.2 $\pm$ 0.4
Den EDL	8.2 $\pm$ 0.2	9.7 $\pm$ 0.4

Again it can be seen that in tibialis anterior muscles there is no increase in total acid phosphatase activity per muscle after denervation but rather a small decrease. In EDL muscles, however, the total acid phosphatase activity per muscle is significantly ($p < 0.02$) increased after denervation.

In paper III it was shown that the increase in specific activity of acid phosphatase 10 days after denervation is more pronounced in the endplate region than outside the endplate region of hemidiaphragm muscles. The endplate region was also shown to be the region of the muscle where segments with high HRP uptake occurred and such segments were shown in paper II to frequently correspond to the segments showing strong staining for acid phosphatase. Taking both biochemical and histochemical data into consideration it is therefore concluded that denervation of skeletal muscle leads to an increase in lysosomal acid phosphatase activity, and that this increase is particularly marked in the endplate region of the muscle fibres where the increase in HRP uptake after denervation also occurs.

No significant changes in acid phosphatase activity were observed in muscles from animals treated with chloroquine (paper VI).

N-acetyl- β -D-glucosaminidase (papers I, III, VI and VII)

N-acetyl- β -D-glucosaminidase is a lysosomal enzyme active against both N-acetylglucosaminides and N-acetylgalactosaminides and is therefore also commonly referred to as β -N-acetylhexosaminidase or just hexosaminidase. Two related forms of hexosaminidase, A and B, have been described and both forms have been shown to be lysosomal although the A form also occurs in the cytosol (see Barret and Heath 1977). The activity of this enzyme has previously been shown to increase in denervated muscles (Wallace and Lewis 1975; Khan and Lewis 1980).

Papers I, VI and VII confirm the increase in the activity of this enzyme after denervation in EDL and tibialis anterior muscles. In all cases the increase in activity is a true increase which is apparent whether expressed as specific activity or as total activity per muscle. In paper I the increase in activity after denervation was shown to be reversible upon reinnervation.

As with acid phosphatase activity, the specific activity of N-acetyl- β -D-glucosaminidase increases more in the endplate region than outside the endplate region after denervation of hemidiaphragms (paper III).

Six days of botulinum toxin-induced muscle paralysis (paper VII) did not affect the activity of N-acetyl- β -D-glucosaminidase in EDL muscles, and this treatment also had no effect on the *in vitro* uptake of HRP (see above). This is in contrast to what was seen for cathepsin D, the other lysosomal enzyme studied (see below), and is also in contrast to the increase in N-acetyl- β -D-glucosaminidase activity which was shown to occur in six day denervated muscles. Eleven days of botulinum toxin-induced muscle paralysis, however, resulted in an increase in the specific activity of N-acetyl- β -D-glucosaminidase in the muscles. In these muscles the HRP uptake per g muscle protein was also increased. As was also noted with the increase in HRP uptake (see above), the increase in N-acetyl- β -D-glucosaminidase activity was not as large as that seen in 11-day denervated muscles. As was also described for the HRP uptake (see above), the N-acetyl- β -D-glucosaminidase activity, when expressed as total activity per muscle, was not significantly higher than that of buffer treated controls but was significantly lower than that of

denervated muscles ($p < 0.01$). The total activity of N-acetyl- β -D-glucosaminidase activity per muscle was, however, about 36% higher in muscles taken 11 days after botulinum toxin poisoning than in the muscles taken six days after poisoning. Although this difference is not statistically significant ($0.05 < p < 0.10$), it suggests that the increase in specific activity of N-acetyl- β -D-glucosaminidase 11 days after poisoning is not only a result of the increased loss of muscle protein.

It is interesting to note that N-acetyl- β -D-glucosaminidase, in contrast to cathepsin D (see below), changes in activity after botulinum toxin-induced muscle paralysis in a way similar to the changes seen in the uptake of the glycoprotein HRP. Since N-acetyl- β -D-glucosaminidase is a lysosomal enzyme which participates in the degradation of the glycan part of sugar-containing macromolecules such as gangliosides, glycosaminoglycans and glycoproteins (see Gregoriadis 1975; Mahuran *et al.* 1985), it is tempting to speculate that the increase in activity of this enzyme is related to the increase in uptake of sugar-containing molecules. Speaking against this possibility, however, is the observation in paper III that the specific activity of N-acetyl- β -D-glucosaminidase increases also outside the endplate region of denervated hemidiaphragms, where no increase in HRP uptake was observed. Previous work has also shown that the specific activity of this enzyme increases in soleus and EDL muscles after neural application of tetrodotoxin (Boegman and Oliver 1980; Boegman and Scarth 1981a), a treatment which did not cause any increase in the uptake of ^3H -inulin or HRP in these muscles (Boegman and Scarth 1981b).

Chloroquine treatment (paper VI) did not cause any change in the activity of N-acetyl- β -D-glucosaminidase in innervated EDL or tibialis anterior muscles. In denervated muscles a small increase in the specific activity was observed after chloroquine treatment, but this increase reached statistical significance only in denervated EDL muscles ($p < 0.05$) and the increase was at least partly due to the slight decrease in protein content observed in denervated muscles after chloroquine treatment.

Cathepsin D (papers I, II, III, VI and VII)

Cathepsin D is often considered to be the major acid proteinase of many animal tissues (Barret 1969; Barret 1977) and its lysosomal localization has been thoroughly established by cell fractionation methods and by immunohistochemical staining (see Dean and Barret 1976; Barret 1977). With regard to skeletal muscle tissue this enzyme has been shown to be able to degrade both purified actin and myosin, as well as the actin and myosin contained in the structural configuration of myofilaments and myofibrils (Bird *et al.* 1977; Schwartz and Bird 1977). Cathepsin D has also been implicated to play a role in conditions of muscle wasting (see Weinstock and Iodice 1969; Bird *et al.* 1978; 1980; Millward 1980).

In the present work the cathepsin D activity of muscles was found to increase in all experimental conditions studied, *i.e.* after denervation (papers I, II, III, VI and VII), after

botulinum toxin-induced muscle paralysis (paper VII) and after chloroquine treatment (paper VI). In all cases the increase is evident whether expressed as specific activity or as total activity per muscle.

In hemidiaphragm muscles the specific activity of cathepsin D, like that of the other lysosomal enzymes studied, was shown to increase more in the endplate region than outside the endplate region 10 days after denervation (paper III).

In two of the experimental models used [6 days of botulinum toxin-induced muscle paralysis (paper VII) and 22 days of chloroquine treatment (paper VI)], the cathepsin D activity of muscles was shown to increase without any concomitant increase in HRP uptake by the muscles. This clearly indicates that at least certain lysosomal enzymes in muscle are regulated at least in part independently of endocytic activity.

In denervated muscles atrophy may proceed despite increased synthesis of total protein (see Obinata *et al.* 1981), suggesting that protein loss is due mainly to increased protein catabolism. A number of different proteolytic enzymes have been shown to exist in skeletal muscle, only some of which are localized in lysosomes (see Bird *et al.* 1980; Millward 1980; Obinata *et al.* 1981; Dahlman *et al.* 1984). With regard to the possible role of cathepsin D as one of these proteolytic enzymes involved in the degradation of muscle proteins (see above), it is interesting to note that the increase in activity of this enzyme 6 days after botulinum toxin poisoning was accompanied by a considerable decrease in muscle protein content (paper VII). Similarly, the chloroquine-induced increase in cathepsin D activity of denervated muscles was accompanied by a small decrease in muscle protein content (paper VI). This decrease in protein content was, however, rather small compared to the large increase in cathepsin D activity measured in the muscle homogenates. In innervated muscles from chloroquine-treated animals the increase in cathepsin D activity occurred without any significant changes in muscle protein content. In muscles from chloroquine-treated animals it may, however, be difficult to correlate the enzyme activity measured in muscle homogenates to the activity which occurs *in vivo* and which may be related to the degree of muscle protein loss. Although cathepsin D is not inhibited by chloroquine *in vitro* (Wibo and Poole 1974), this drug may induce changes in the lysosomes *in vivo* which could impair the activities of various lysosomal enzymes. Such changes may include an increase in the intralysosomal pH which has been shown to occur in the presence of chloroquine (see de Duve 1983; Reeves 1984; Maxfield 1985). In this context it should also be noted that the activity of cathepsin D falls away steeply at pH values above 5.5 (Barret 1980).

An earlier study of the effect of chloroquine treatment on cathepsin D activity in chicken muscles revealed no change in activity after 7 days of treatment (Stauber *et al.* 1981a). The apparent discrepancy between the results of that study and the results of the present work (paper VI) may be due to differences in species studied, dosage and time period of drug treatment.

The increase in cathepsin D activity which occurs after denervation was shown to be reversible upon reinnervation (paper I).

Time course of changes in endocytic activity and cathepsin D activity after denervation (papers I and II)

In paper II a comparison of the time course of changes in endocytic activity and the changes in the activity of cathepsin D after denervation was made. This comparison indicated that the endocytic activity increases more rapidly after denervation than does the activity of the lysosomal enzyme cathepsin D. This is in accordance with the working hypothesis that increased endocytic activity may be one of the mechanisms for lysosomal activation in skeletal muscle.

In the light of the results obtained in later studies, however, this comparison may require some further discussion. Firstly, it was shown that the cathepsin D activity of muscles may increase without any increase in endocytic activity (papers VI and VII). Secondly, it was shown in paper III that although the cathepsin D activity after denervation increases more in the area of the muscle fibre where the increased HRP uptake occurs (the endplate region), some increase in cathepsin D activity occurs also in other areas of the muscle fibre. It may thus be concluded that only part of the increase in cathepsin D activity after denervation is directly related to the increase in endocytic activity. Without taking this into consideration, a detailed comparison of the time course of the increases after denervation may not be justified.

Lysosomal enzyme activities in the endplate region of denervated muscle fibres (paper III)

The specific activities of the three lysosomal enzymes acid phosphatase, N-acetyl- β -D-glucosaminidase and cathepsin D were shown to be increased in both the endplate region and extrajunctional regions of 10-day denervated mouse hemidiaphragms. For all three enzymes, however, the specific activities were significantly higher in the endplate region than in the peripheral regions. In innervated control hemidiaphragms no differences were observed between specific activities in the endplate region and peripheral regions. The endplate region was also shown by biochemical and morphological methods to be the locus of the increased uptake of HRP in denervated muscles.

The results on the specific activities of the different lysosomal enzymes in the endplate region and peripheral regions of denervated hemidiaphragms are somewhat at variance with results reported by Maskrey *et al.* (1977). These authors showed that the increases in the specific activities of acid proteinase, cathepsin B₁ and α -glucuronidase due to denervation are the same in the endplate region and peripheral regions of rat hemidiaphragms four days after denervation. The apparent discrepancy between these results and the results reported in paper III may be due to factors such as species differences and differences in the lysosomal enzymes studied. The only common enzyme between the studies is the acid proteinase of Maskrey *et al.* (1977), which should correspond to the cathepsin D studied in paper III. This enzyme was also the one which showed the least significant difference between specific activity in the endplate region and

peripheral regions of 10-day denervated hemidiaphragms.

The most likely explanation for the apparent discrepancy is, however, that the studies were carried out at different time periods after denervation. The endocytic activity of muscle fibres increases 2-4 days after denervation, judged from biochemical studies (see above). This leads to the formation of segments with high endocytic activity in the endplate region, which can be demonstrated morphologically 3-6 days after denervation (papers II and III). The increase in lysosomal enzyme activities in the endplate region of denervated muscle fibres is also expected to develop during these time periods. It may therefore be that at four days after denervation there is no difference between lysosomal enzyme activities of endplate and peripheral fibre regions, whereas 10 days following denervation this difference has developed.

MORPHOLOGICAL STUDIES

Histochemical and gross morphological studies of horseradish peroxidase uptake in denervated muscle fibres (papers II and III)

In an earlier morphological study on the HRP uptake in denervated muscle fibres (Libelius *et al.* 1979a) longitudinal sections showed that this uptake was localized segmentally in the muscle fibres. In cross sections the HRP deposits were often seen to be circularly distributed with sparing of more central and peripheral parts of the muscle fibres.

In paper II these results were confirmed using a histochemical technique for staining of peroxidase in sections of fixed tibialis anterior muscles. The observed peroxidase activity appeared to be contained within circumscribed bodies giving a granular appearance to the segments. In longitudinal sections the HRP-positive bodies were distributed in a spindle-shaped fashion, leaving the most peripheral and central regions of the fibres devoid of staining. At the ends of such segments the HRP-positive bodies became more centrally located, frequently smaller in size, and gradually faded away. In transverse sections the HRP-positive bodies were usually seen distributed in a ring or occasionally centrally in the fibres, depending on at which level the segments had been sectioned. The length of individual segments varied a great deal from about 100-600 μm .

The described segments were not observed in sections of fresh-frozen denervated muscles. The reason for this may be that fixation is required for preserving the localization of the HRP in the tissue. If the tissue is not fixed, freezing may induce a diffuse dispersion of the enzyme into the unfixed tissue or the enzyme may become dispersed or extracted during staining.

In sections of fixed muscles the segments showing strong peroxidase staining frequently also showed positive staining for the lysosomal enzyme acid phosphatase in consecutive serial sections (paper II). This suggests that the observed peroxidase staining is due to HRP which has accumulated in the lysosomal system of the muscle fibres. Similar segments showing positive

staining for acid phosphatase were also observed in denervated muscles from animals which had not been injected with HRP, suggesting that the segmental increase in lysosomal activity is a result of denervation and not dependent on the presence of HRP. The specificity of the staining reaction for acid phosphatase was checked by pre-treating sections with 10 mM NaF or by incubating in the presence of NaF. Such control sections showed no staining for acid phosphatase.

In order to study the time course of the appearance of HRP segments after denervation a morphometric study was performed (paper II). For this study muscles from animals injected with HRP were taken at different times after denervation. After fixation approximately the proximal 1/5 and the distal 1/3 of the muscles were cut away. The remaining pieces were sectioned transversely starting from the proximal end and 16 sections of 10 μ m thickness and separated by 140 μ m were taken from each muscle. In each of the sections the number of fibres with HRP-positive inclusions, distributed in a ring or centrally located in the fibres, were then counted in a microscope by complete visual scanning of the sections at a magnification of $\times$ 160-400.

The results of the morphometric study showed that the main increase in the average number of HRP-positive fibres per cross section occurred between days 3 and 6 after denervation. Whether this increase represents a gradual increase in the number of fibres containing segments or an increase in the length of the segments is not entirely clear. The cross sections examined were separated by 140 μ m and, since the length of segments varied considerably (at least 100-600 μ m), some segments will never have been sectioned whereas others will have been sectioned several times. No quantitative study was carried out on longitudinal sections and therefore the average length of segments is not known. It is also not known whether or not the average length changes with time after denervation.

It is interesting to note that the uptake of extracellular markers increases rather abruptly 2-4 days after denervation as judged by the biochemical studies (see above). The histochemically detectable uptake, however, occurs somewhat later. The reason for this apparent discrepancy may be that early after denervation the uptake occurs into structures which are not large enough to be detectable by histochemical methods. This may also be the reason why in innervated muscles, although they show a biochemically measurable uptake of HRP, this HRP uptake cannot be visualized using the histochemical technique.

The morphometric study also revealed that the HRP-positive segments occur with an uneven distribution in denervated tibialis anterior muscles (paper II). In the sectioned part of the muscles, there was a significant decrease in the number of HRP-positive fibres per section from proximal to more distal sections. This observation, together with the observation from preliminary ultrastructural studies that synaptic folds were frequently present in the vicinity of segments with HRP uptake, prompted an investigation of the possible relationship between segments with high HRP uptake and the endplate region of denervated muscle fibres.

The study of the distribution of HRP-containing segments in denervated muscle was performed on denervated mouse hemidiaphragm muscle since this is a thin and wide muscle with endplates localized in a narrow central band (Waser and Lüthi 1956).

The results reported in paper III clearly show that the HRP accumulation 8-12 days after

denervation occurs in segments which are located near the endplate region of the muscle fibres. In accordance with the observations reported in paper II no peroxidase-positive segments were seen 2 days after denervation and 4 days after denervation only occasional segments were observed. Later studies (unpublished) have shown that the pattern of HRP uptake in 7 weeks denervated hemidiaphragms is very similar to that observed in 8-12-day denervated muscles.

Ultrastructural studies of horseradish peroxidase uptake in denervated muscle fibres (papers III, V and VI)

The ultrastructural studies were carried out mainly on muscle fibres dissected from denervated tibialis anterior muscles. Muscles were taken at different times (4-14 days) after denervation and at various times (15 min-24 hr) after injection of HRP (paper V). The effects of chloroquine treatment on the ultrastructure of muscle fibres and in particular on the ultrastructural appearance of HRP-containing segments were also studied (paper VI).

Since the segments with high peroxidase content are unevenly distributed in denervated muscles and since blocks of muscle tissue do not give homogeneous staining conditions (Libelius *et al.* 1979a), a method for dissecting and staining single muscle fibres was developed. Segments with high peroxidase content could easily be detected in the light microscope and even, after some practice, under a dissecting microscope. This method proved to be convenient for preparing a relatively large amount of well-stained material for ultrastructural studies. A possible risk with the method is that some mechanical damage may occur during the dissection and handling of the fibres. To minimize this the muscles were carefully fixed by perfusion, injection of fixative into the muscles *in situ* and immersion in fixative for 2 hr. The fixative also contained a higher aldehyde concentration (1% formaldehyde, 2% glutaraldehyde) than used previously (1% formaldehyde, 1% glutaraldehyde see Libelius *et al.* 1979a). All handling of the fibres was carried out under a dissecting microscope and care was taken to apply any instruments only to the ends of the fibres, avoiding touching the area of the segment.

Localization of the uptake

The ultrastructural studies (papers III and V) confirmed the relationship of the HRP-containing segments to the endplate region since post-synaptic folds or remnants thereof were seen in the vicinity of a large proportion of the segments (12 out of 68 segments studied in paper V). The segments were, however, larger than the endplate region and extended far beyond it. The uptake also did not seem to occur directly from the folds or other parts of the sarcolemma, but was usually localized deeper in the muscle fibres.

The most conspicuous feature of the segments was the presence of large peroxidase-containing phagosomes. These occurred with a large variation in size and shape, frequently extending over

the length of several sarcomeres.

Lysosomal nature of horseradish peroxidase-containing phagosomes

The peroxidase-containing phagosomes also appeared to participate in autophagic processes since they were frequently seen to contain undegraded material; occasionally, structures resembling mitochondria could be identified inside phagosomes (paper V). Phagosomes apparently in the process of engulfing cytoplasmic components were also frequently observed. These observations suggest that the peroxidase-containing phagosomes also have lysosomal functions and this is in agreement with the histochemical observations which showed similar distributions of peroxidase and acid phosphatase staining in denervated muscle fibres (paper II).

That at least some of the peroxidase-containing phagosomes are part of the lysosomal system of the muscle fibres is also strongly suggested by the results of the chloroquine study (paper VI). A number of previous ultrastructural studies have shown that this drug induces the formation of myeloid bodies and autophagic vacuoles in skeletal muscle fibres (Garcin *et al.* 1964; Rewcastle and Humphrey 1965; MacDonald and Engel 1970; Gérard *et al.* 1973; Drenckhahn and Lüllmann-Rauch 1979; Lüllmann-Rauch 1979; Schmalbruch 1980; Stauber *et al.* 1981a; 1981b; Trout *et al.* 1981c). The ultrastructural changes may be due to two main effects of the drug. Firstly, since the drug is a lipophilic weak base it will penetrate into lysosomes where it, because of the low pH, becomes charged and thereby trapped inside the lysosomes. This accumulation of chloroquine in the lysosomes is believed to interfere with their functions (see de Duve *et al.* 1974). Secondly, due to electrostatic and hydrophobic attractions the drug interacts with various types of membrane phospholipids and this interaction is believed to alter the properties of the phospholipids in such a way as to make them more resistant to hydrolysis (see Lüllmann-Rauch 1979).

The peroxidase-containing phagosomes in segments of denervated muscle fibres from animals treated with chloroquine were frequently seen to contain very large amounts of undegraded material, which is as expected if these phagosomes are of a lysosomal nature and participate in autophagic processes (paper VI).

Mechanism of horseradish peroxidase uptake

A previous ultrastructural study (Libelius *et al.* 1979a) suggested that the uptake of HRP occurs by endocytosis (micropinocytosis) from t-tubuli. The biochemical evidence discussed above is also strongly in favour of a (receptor-mediated) endocytic uptake mechanism. It has been pointed out, however, that much of the available data on pinocytosis may also be explained without a net translocation of membrane (see Schneider *et al.* 1979b; Lloyd 1980). Instead of transport of substances via membrane vesicles the transfer of substances could be imagined to occur through a

series of compartments transiently interconnected by alternate fusion-fission events or even more permanently interconnected by narrow channels fitted with appropriate locks. Conventional electronmicroscopy, as used in the present work, cannot differentiate between these possibilities since it only provides a static two-dimensional picture of a dynamic three-dimensional process. Examination of serial sections would be required for three-dimensional studies and pulse labelling followed by different chase periods would be required in order to reveal the dynamic nature of the process. Due to the rapidity of endocytic processes, chase experiments of high resolution are probably impossible to carry out in whole tissues such as muscle since the tissue can probably not be fixed rapidly enough.

The results of the main ultrastructural study (paper V) are compatible with the suggestion that the uptake of HRP occurs from t-tubuli. This is indicated by the localization of the uptake deep in the muscle fibres rather than immediately under the sarcolemma and by the vivid proliferation of the t-system in the regions where the HRP uptake occurs. T-tubular proliferation was seen as elaborate networks of vesicular profiles, t-tubular profiles with an enlarged appearance and longitudinally oriented t-tubuli. T-tubular profiles also appeared to encircle cytoplasmic areas and/or cytoplasmic bodies.

Some observations made in paper V may suggest a component of passive filling by diffusion of some phagosomes. One such observation is that some phagosomes were seen to have "tails" which could suggest a communication with the t-system. Large phagosomes could also be seen filled with peroxidase already 10-15 min after the animal was injected with HRP. It is difficult to know, however, if this could also be explained by endocytic activity since it is not known how much HRP is required inside a phagosome for it to appear "filled" with peroxidase. Peroxidase-containing phagosomes were, however, still present 24 hr after administration of HRP. After this time period many t-tubuli contained no peroxidase activity suggesting that phagosomes in free communication with t-tubuli should also be empty, unless the HRP was strongly adsorbed to the phagosome membrane. Unpublished studies on denervated hemidiaphragms have also shown the presence of segments 48 hr after HRP administration and after this time period the plasma peroxidase concentration had decreased to $< 1 \mu\text{g/ml}$, again suggesting that HRP is not completely free to diffuse passively into and out of the phagosomes seen in the segments.

The uneven labelling of t-tubuli seen 10-24 hr after HRP injection (paper V) suggests the possibility that some HRP may be excreted from the muscle fibres. This is in accordance with biochemical results (paper IV) showing that muscles loaded with HRP lose some activity after continued incubation in the absence of marker and that some of the lost activity can be recovered in the incubation solution.

Many macromolecules which enter cells by receptor-mediated endocytosis have been suggested to do so via coated pits in the plasma membrane which after internalization are seen as coated vesicles (see *e.g.* Goldstein *et al.* 1979; Salisbury *et al.* 1983). This route of internalization is, however, not generally accepted. Some studies have suggested that coated pits are important for concentrating receptors with attached ligand, and that the coated pits then go through a non-communicative phase in which they are functionally out of communication with the cell surface but are not separated from it. Such "cryptic" coated pits are then suggested to lead to the formation of intracellular uncoated vesicles (receptosomes) by invagination of membrane adjacent to the coated region (see Willingham *et al.* 1981; Willingham and Pastan 1984; Pastan and Willingham 1985).

In the present study (paper V) coated vesicles were rare in the segments, although such vesicles were observed in the previous ultrastructural study (Libelius *et al.* 1979a) and in studies of HRP uptake in cultured myotubes (Bursztajn and Libby 1981; Janeczko *et al.* 1985). The apparent absence of coated vesicles in the present study may have many possible explanations. One is that the intense staining reaction for peroxidase inside vesicles may make a thin coat around them difficult to be visualized. Secondly, coated vesicles often seem to be very short-lived structures which lose their coat within 15-60 s after their formation (see Steinman *et al.* 1983; Mellman 1984). Demonstration of such short-lived structures will therefore probably require more rapid fixation than is likely to occur with whole muscle tissue.

Other ultrastructural features in segments of denervated muscle fibres with high horseradish peroxidase uptake (paper V)

Myofibrillar degeneration

A pronounced myofibrillar degeneration was frequently seen in the segments of the muscle fibres where high HRP uptake occurred (papers III and V). Most frequently the degeneration was seen centrally in the muscle fibres, but focal degenerative changes could also be seen in other areas of the segments. The degeneration appeared to progress with time since it was more frequently observed 12-14 days after denervation than 4 days after denervation. Signs of myofibrillar degeneration were also reported in the previous ultrastructural study by Libelius *et al.* (1979a). The cause of the pronounced degeneration is not clear but it may be related to the high endocytic and lysosomal enzyme activities which occur in this area, as suggested by the working hypothesis discussed above. Other possible causes, however, cannot be excluded.

It should be noted that the myofibrillar degeneration which occurs in the segments is frequently very pronounced, often leading to complete loss of cross-striation in large areas of the segment.

This degenerative process is thus much more prominent than the myofibrillar disintegration reported in "simple" denervation atrophy, which appears to occur by a loss of myofilaments from the periphery of individual myofibrils (Pellegrino and Franzini 1963; Shafiq *et al.* 1967; Miledi and Slater 1969; Stonnington and Engel 1973; Tomanek and Lund 1973; Engel and Stonnington 1974). The "simple" atrophy starts early after denervation but its effects are more detectable after one month (Pellegrino and Franzini 1963). In addition to this "simple" atrophy, Pellegrino and Franzini (1963) also described a degenerative autolytic process which occurs during the first two weeks after denervation and which results in loss of striation in areas of the fibres. This process was suggested to be responsible for a gross weight loss of 50%. The degeneration was shown to start by alterations of the Z lines and later filaments were seen to lose their parallel arrangement over the whole depth of the myofibril. The alteration was shown to spread transversely in the fibre along the Z line, from one fibril to the next. The process was found to result in large areas, particularly at the periphery of fibres, containing no contractile material. A single fibre could be affected several times along its length but the disarrangement of the contractile material did not occur in more than two or three sarcomeres at a time (Pellegrino and Franzini 1963). The degeneration which occurs in the segments with high HRP uptake (paper V) was frequently found to be localized in the centre of the fibre; it occurred in the vicinity of the endplate region and it often involved a much larger number of sarcomeres than the degenerative process referred to above.

The myofibrillar degeneration which occurs primarily at the centre of segments produces lesions which ultrastructurally are somewhat similar to those seen in target fibres. Target fibres have previously been described in denervated muscles (Engel 1961) and/or in muscles becoming reinnervated (Dubowitz 1967). This suggests the possibility that target fibres may in some way be related to the segments of muscle fibres which show high endocytic activity.

Golgi apparatus

In the segments with high HRP uptake Golgi profiles were seen relatively frequently (paper V). Occasionally a single electron micrograph could be seen to contain 4-5 different Golgi complexes. These Golgi complexes frequently occurred deep in the muscle fibres and in close proximity to peroxidase-containing phagosomes. In no case, however, were Golgi cisternae seen to contain any peroxidase label.

The Golgi apparatus is believed to play an important role in the processing and sorting of macromolecules which are to be either inserted into the plasma membrane, secreted from the cell or segregated into certain specialized compartments of the cell such as lysosomes (see Farquhar 1978; Goldfischer 1982). In normal innervated muscle fibres Golgi complexes are not particularly abundant. When seen they are usually located in the sarcoplasm near the nuclear poles. Golgi complexes have previously been reported to become more numerous in denervated muscle fibres (Gori 1972; Tomanek and Lund 1973; Engel and Stonnington 1974; Cullen and Pluskal 1977).

The abundance of Golgi profiles, the high endocytic activity and the prominent myofibrillar

degeneration seen in the endplate region of denervated muscle fibres leaves a strong impression suggesting that this part of the muscle fibre is undergoing a very significant change in its function. From having been a part of a fibre participating in the mechanical activity of the muscle, the endplate region appears to change after denervation into a fibre segment involved more in ingestion of extracellular material, membrane turnover and possibly in secretory activities. It is tempting to speculate that these new activities are all somehow aimed at establishing optimal conditions for the formation of new functional synaptic contacts in the endplate region.

Sarcoplasmic reticulum

Membrane-limited bodies which were identified as enlarged components of the sarcoplasmic reticulum could frequently be observed in the segments with high HRP uptake (paper V). That these bodies were indeed derived from the sarcoplasmic reticulum was suggested by the fact that they could frequently be seen in close apposition to t-tubuli. They also had a distinctive fine-granular appearance which is a characteristic of the sarcoplasmic reticulum doubly fixed with glutaraldehyde and osmium (Engel and MacDonald 1970). These components of the sarcoplasmic reticulum were encountered immediately under the sarcolemma and deeper in the fibres in the vicinity of areas showing t-tubular proliferation and peroxidase-containing phagosomes. An over-development and enlargement of the sarcoplasmic reticulum has been reported previously in denervated muscles (Pellegrino and Franzini 1963; Muscatello *et al.* 1965; Shafiq *et al.* 1967; Gori 1972; Stonnington and Engel 1973; Tomanek and Lund 1973).

Many previous studies have suggested that portions of the sarcoplasmic reticulum may contain lysosomal enzymes and participate in the formation of autophagic vacuoles (Pearce 1966; Christie and Stoward 1977; Schmalbruch 1980). The presence of lysosomal enzymes, such as acid phosphatase and cathepsins B and D, in parts of the sarcoplasmic reticulum has been confirmed by both biochemical studies (Stauber and Bird 1974) and cytochemical staining at the ultrastructural level (Pearce 1966; Seiden 1973; Bird *et al.* 1977; 1978; Libelius and Lundquist 1978; Trout *et al.* 1979a; 1979b; Spicer *et al.* 1980). These observations raise the possibility that the enlarged components of the sarcoplasmic reticulum seen in the segments with high HRP uptake are also part of the lysosomal system which by other methods has been shown to be well developed in these segments (see above). Staining for lysosomal enzymes at the ultrastructural level is, however, required in order to establish this.

Other membrane limited bodies

Other membrane-limited bodies observed in the segments with high HRP uptake were of multivesicular appearance, usually containing peroxidase staining, and bodies of similar size and shape

to the peroxidase-containing phagosomes but with weaker staining or unstained (paper V). The role of these bodies is unclear. Possibly the weaker stained or unstained bodies represent phagosomes/vacuoles which were formed before HRP injection or after the disappearance of extracellular HRP. Further information about the role and function of these bodies may be gained by studying the endplate region of denervated muscle fibres from animals not injected with HRP.

Other ultrastructural features of muscle fibres from chloroquine-treated animals (paper VI)

As discussed above, segments with high HRP uptake in denervated muscle fibres from animals treated with chloroquine contained phagosomes with large amounts of undegraded material. Such fibres, as well as other parts of denervated muscle fibres and innervated fibres, also contained chloroquine-induced autophagic vacuoles, devoid of peroxidase staining, and myeloid bodies. Very large vacuoles like those observed in the study by MacDonald and Engel (1970) were not observed. This may be due to species differences, differences in the period of drug treatment and the fact that the present work was carried out on tibialis anterior muscles which contain a mixture of different fibre types. Previous studies have shown that although both predominantly "white" and predominantly "red" muscles are ultrastructurally affected by chloroquine treatment, predominantly "red" muscles are more severely affected (Smith and O'Grady 1966; MacDonald and Engel 1970; Schmalbruch 1980; Trout *et al.* 1981c). The reasons for choosing tibialis anterior muscles for the present work were that this muscle had been used for our previous ultrastructural work and that, despite quantitative differences, predominantly "white" and predominantly "red" muscles are affected in a qualitatively similar way by chloroquine treatment (MacDonald and Engel 1970; Trout *et al.* 1981c).

In some muscle fibres from chloroquine-treated animals mitochondria were somewhat swollen with separated cristae. Since this was never observed in muscle fibres from animals not treated with chloroquine this was considered as another effect induced by the drug. Similar changes in the ultrastructural appearance of mitochondria have previously been reported in muscles from chloroquine-treated rabbits (Aguayo and Hudgson 1970) and in chloroquine-treated foetal mouse hearts in culture (Ridout *et al.* 1978).

GENERAL DISCUSSION

"Sometimes I sits and thinks, and sometimes I only sits"

(Mr Martin Post: Atlantic College Yearbook, 1973-1975)

The results presented in this thesis have extended the observation that denervated muscles have increased endocytic activity and shown that this increase in endocytosis occurs primarily near the endplate region of denervated muscle fibres. Results have also been presented which show that the uptake of HRP in skeletal muscle occurs by a receptor-mediated endocytic process which involves receptors recognizing mannose residues in glycoproteins. The physiological and/or pathophysiological significance of these observations is presently unknown but may be related to trophic interactions between nerve and muscle.

POSSIBLE CELL BIOLOGICAL RELEVANCE OF ENDOCYTOSIS IN SKELETAL MUSCLE

Intercellular communication is of major importance for a correct formation and function of the nervous system and for its co-operation with innervated organs. This communication is likely to operate in both directions, *i.e.* from the nerve to the effector cell and from the effector cell back to the nerve. Skeletal muscle, with its motoneuronal innervation, offers a suitable preparation for studies of intercellular communication since the muscle is a highly specialized tissue which is critically dependent on the motor nerve input for carrying out its physiological functions. If this input is lost the muscle becomes useless in terms of its physiological functions, unless it can become reinnervated. A normal innervated muscle fibre will not accept any additional neuronal input (see Grinnell and Herrera 1981). A denervated muscle fibre, on the other hand, if approached by a motoneurone will establish a new functional synaptic contact. Since this is the only way for a muscle fibre to regain its physiological functions it is reasonable to assume that some of the changes that occur in a denervated muscle are aimed at attracting a new motoneurone and at establishing optimal conditions for the formation of a new functional synaptic contact. The changes which occur in muscle after denervation are believed to result from the disappearance of the trophic influence of the nerve on the muscle. The nature of this trophic influence is not clear, but activity in the muscle is believed to be of importance and unspecified chemical factors have also been suggested to play a role.

Membrane changes in denervated muscle fibres

Many of the changes which have been described in denervated muscle fibres are related to the properties of the muscle fibre membrane. In normal innervated muscle fibres sensitivity to acetylcholine is limited almost exclusively to the endplate region (del Castillo and Katz 1955) and the acetylcholine receptors located here are very stable with a turnover half-life of about 13 days (see Fambrough 1979). After denervation, muscle fibres become sensitive to acetylcholine also in extrajunctional fibre regions (Axelsson and Thesleff 1959). Extrajunctional acetylcholine receptors have been shown to have a turnover half-life of only 6-35 hr (see Fambrough 1979). The total number of acetylcholine receptors per muscle increases 5-50 times after denervation (see Fambrough 1979).

Another membrane protein which undergoes changes after denervation is the sodium channel which operates in the generation of action potentials in the muscle fibres. In normal innervated muscle this channel is blocked by tetrodotoxin but after denervation new sodium channels appear which are resistant to the blocking action of tetrodotoxin (Redfern and Thesleff 1971). The appearance of these new sodium channels after denervation was shown to be dependent on *de novo* protein synthesis (Grampp *et al.* 1972).

The above described changes in the membrane properties of muscle fibres which occur after denervation suggest that denervation is associated with an increased synthesis and degradation of certain membrane proteins. These changes may conceivably be dependent on an increase in exocytic and endocytic activity of the muscle fibre since integral membrane glycoproteins, such as the acetylcholine receptor, are believed to reach the cell surface after synthesis by a pathway similar to that taken by secretory proteins (see Fambrough and Devreotes 1978; Pumplin and Fambrough 1982). In line with this it has also been shown that in normal endplates and in autoimmune myasthenia gravis acetylcholine receptors are degraded following internalization by endocytic uptake (Engel and Fumagalli 1982; Fumagalli *et al.* 1982). In paper I a temporal correlation was also observed between changes in the tetrodotoxin sensitivity of muscle fibre action potentials and changes in the endocytic activity of muscles after denervation and during reinnervation.

The appearance of extrajunctional acetylcholine receptors after denervation may be related to the ability of the muscle fibre to receive innervation at sites distant from the original endplate (see Lømo 1980; Grinnell and Herrera 1981). Other changes which occur in the membrane of denervated muscle fibres have also been suggested to play a role in the capacity for cell-cell interactions associated with denervation. Such changes include alterations in surface membrane carbohydrate composition and glycosyltransferase activities (*e.g.* Jeffrey and Appel 1978; Leung *et al.* 1982; Leung *et al.* 1984) and the appearance of membrane glycoproteins, such as the Thy-1 glycoprotein (Booth *et al.* 1984) and neural cell adhesion molecule (N-CAM) (Covault and Sanes 1985; Rieger *et al.* 1985), which are normally expressed on embryonic myoblasts and/or myotubes. Again the insertion of such new components into the membrane may well require increased exocytic and endocytic activities of the muscle fibres.

For many of these described changes in the muscle fibre membrane the alterations after denervation appear to occur throughout the length of the muscle fibre. How this can be accommodated with the observation that the increase in endocytic activity after denervation occurs mainly in the endplate region is not clear. Possibly the high endocytic activity in the endplate region is related mainly to changes which occur in this area of the muscle fibre. In this context it is interesting to note that after denervation the packing density of receptors at former endplates remains comparable to that at normal neuromuscular junctions (see Fambrough 1979). Following denervation, however, the half-life of these junctional receptors decreases towards that reported for extrajunctional receptors (Levitt *et al.* 1980). Thus, after denervation the turnover of acetylcholine receptors at the endplate region may well require higher rates of exocytosis/endocytosis than that required by other fibre regions. The rate of protein synthesis has also been shown to be particularly high in the endplate region of mouse hemidiaphragms 11 days after denervation (Kimura and Kimura 1973).

The accumulation of neuronal cell adhesion molecule (N-CAM) after denervation was also shown to occur in largest amounts near denervated endplates (Covault and Sanes 1985). This accumulation was, however, mainly associated with interstitial deposits of N-CAM while the post-synaptic membrane itself was only slightly richer in N-CAM than adjacent extrasynaptic areas (Covault and Sanes 1985). The accumulation of N-CAM near denervated endplates was suggested to play a role in guiding axons to the denervated endplate (Covault and Sanes 1985) where new synapses preferentially form (see Grinnell and Herrera 1981).

Synapse formation

In the developing central nervous system endocytic activity in post-synaptic cells appears to be important for establishing synaptic contacts. In the cerebellum a large increase in the number of pinocytotic vesicles are observed in Purkinje cells just prior to the formation of synapses between parallel fibres and Purkinje cells (Palacios-Prü *et al.* 1981). In this system it was also shown that the membrane of the newly formed parallel fibres is rich in a specific class of glycoprotein which binds to concanavalin A and which disappears in the period when synaptogenesis is taking place (Zanetta *et al.* 1978). These and other observations resulted in the hypothesis that the glycans of the concanavalin A-binding glycoproteins on parallel fibres interact first with specific receptor molecules found on the membrane of their target cells. This recognition is suggested to be followed by endocytosis of the glycoprotein-receptor complex into the target cells and migration towards the lysosomes, where the glycoproteins are at least partially degraded (see Dontenwill *et al.* 1983). Support for a role of endocytic activity in the formation of neuromuscular junctions has also been presented in ultrastructural studies on the formation of neuromuscular junctions in tissue culture (James and Tressman 1969; Bird and James 1974).

Whether or not the observed endocytic activity in the endplate region of denervated muscle fibres has any relationship to the observations on synapse formation described above can at this

stage only be a matter of speculation. It may be important in this context to remember that the processes operating in embryonic synapse formation may differ from those operating during reinnervation. Once functional and lasting synaptic contacts have been formed between nerve and muscle a kind of "imprinting" has been made which remains even after denervation. This "imprinting" may be seen in the maintained high density of acetylcholine receptors in the endplate region of denervated muscle fibres and in the maintained synaptic specialization of the muscle fibre basal lamina (see Sanes *et al.* 1978; Sanes and Hall 1979; Anglister and McMahan 1984).

Nerve-derived trophic factor

A glycoprotein factor capable of ameliorating atrophy of denervated rat skeletal muscle has recently been partially purified from sheep sciatic nerve extracts (Davis *et al.* 1985). How this trophic factor works is still unknown, but to exert its effects on muscle fibres it may well have to be taken up by an endocytic mechanism.

Motoneurone growth factor

Considerable evidence has accumulated suggesting that skeletal muscle cells, especially denervated and foetal, secrete a growth factor for motoneurons (see Slack *et al.* 1983). This factor may be responsible for the occurrence of sprouting of motoneurons after partial denervation and after poisoning with botulinum toxin. Extracts of denervated or foetal muscles have been shown to stimulate the growth of motoneurons in culture (*e.g.* Dribin and Barrett 1980; Henderson *et al.* 1981; Smith and Appel 1983; Nurcombe *et al.* 1984). Antisera against a protein secreted by denervated rat muscles were recently shown to suppress botulinum toxin-induced terminal sprouting in the mouse (Gurney 1984). Interestingly, serum from some patients with amyotrophic lateral sclerosis (ALS) was also shown to suppress botulinum toxin-induced terminal sprouting in mice, and this inhibitory activity was mediated by an immunoglobulin recognizing the above-mentioned protein secreted by denervated rat muscles (Gurney 1984; Gurney *et al.* 1984). These results indicate that some cases of ALS may be due to a deficiency in the function of a motoneurone growth factor as previously suggested by Appel (1981).

The denervated endplate region may be particularly important for the production of this motoneurone growth factor since destruction of the endplate region before partial denervation was shown to prevent sprouting of the remaining motoneurons (Keynes *et al.* 1983). In the light of these observations it is conceivable that the HRP uptake in the endplate region of denervated muscle fibres labels an area of the fibres which is active in the production and secretion of this motoneurone growth factor.

Fibrillation potentials in denervated skeletal muscle

Irregularly discharging fibrillations occur in denervated muscles and are due to action potentials triggered by discrete, spontaneous and randomly occurring depolarizations (see Thesleff 1982). Such fibrillations are particularly prevalent in the mouse diaphragm where they begin during the third day of denervation and reach a maximum 9-12 days after sectioning of the nerve. The fibrillation potentials start in the central part (endplate region) of the muscle fibres and are believed to be caused by spontaneous action potentials within t-tubuli (Purves and Sakmann 1974; Smith and Thesleff 1976). The morphological origin of irregularly discharging fibrillations is therefore the same as that of HRP uptake in denervated muscles. This suggests the possibility that the processes triggering such fibrillations may be related to endocytic activity.

Possible role of muscle tissue in the metabolism of extracellular substances

The estimated rate of endocytic uptake of extracellular fluid into innervated EDL muscles was found to be large enough to turn over the entire extracellular volume of the muscles in just over 20 hours. By virtue of the overall mass of muscle in the body this level of endocytic activity raises the possibility that skeletal muscle may contribute to the metabolism of extracellular substances. Considering also that some substances may be taken up specifically by receptor-mediated uptake processes this possibility becomes even more plausible.

Possible functions of receptors recognizing mannose residues in glycoproteins

The present work has shown that the uptake of HRP in skeletal muscle occurs by a receptor-mediated endocytic process which involves receptors recognizing mannose residues in glycoproteins. Recently a mannose-6-phosphate receptor has also been shown to occur in skeletal muscle (Reuser *et al.* 1984; Salminen 1984). Both mannose-6-phosphate receptors and mannose receptors have in other cell systems been implicated in intra- and/or intercellular transport of lysosomal enzymes (*e.g.* Sly and Stahl 1978; Sly and Fischer 1982; Shepherd *et al.* 1983; Creek and Sly 1984; Gabel *et al.* 1984; Sahagian 1984; Shepherd and Stahl 1984; Stahl *et al.* 1984). That skeletal muscle fibres in certain circumstances may acquire lysosomal enzymes which originate from other cell types has also been suggested (Salminen 1985; Schwartz *et al.* 1985).

There are, however, also a number of other possible functions for the mannose receptors. One such possibility is that they could participate in the segregation and uptake of plasma membrane glycoproteins terminating in sugars which bind to the receptors (Shepherd and Stahl 1984). Results in accordance with such a function were obtained by Chung *et al.* (1984) who found that swainsonine treatment of macrophages blocks the uptake of mannose-terminated glycoproteins. Swainsonine is an alkaloid which inhibits the enzyme α -mannosidase II which is involved in the

processing of glycoproteins in the Golgi apparatus (Elbein *et al.* 1981; Tulsiani *et al.* 1982). Treatment with swainsonine brings about the accumulation of unprocessed mannose-terminated oligosaccharide chains on both secretory and membrane glycoproteins. The mannose-terminated membrane glycoproteins formed in the presence of swainsonine may interact with the mannose receptor, thus preventing the receptor from becoming engaged in the uptake of extracellular glycoproteins (Chung *et al.* 1984).

Mannose receptors have also been suggested to participate in the uptake into, *e.g.* non-parenchymal hepatic cells, of immunoglobulin M (IgM) when complexed with antigen (Day *et al.* 1980). Native IgM molecules contain mannose-terminal oligosaccharides which may be buried within the quaternary structure of the molecule and thus inaccessible to mannose receptors and rapid clearance by endocytosis. Exposure to antigen is believed to cause a conformational change in the molecule which results in exposure of mannose residues. These can then interact with mannose receptors which mediate endocytic uptake into various cells (Day *et al.* 1980).

Mannose-specific binding of HRP to cells in sections of endocrine organs from rats has also been shown to be inhibited by various glycoprotein hormone preparations (Straus 1983b).

Lysosomes in skeletal muscle

Normal innervated skeletal muscle contains biochemically measurable activities of lysosomal enzymes. In ultrastructural studies it has, however, been extremely difficult to demonstrate the presence of lysosomes in muscle fibres. Even in denervated or diseased muscles lysosomes are not particularly prevalent in muscle fibres. The present work has shown, by both biochemical and histochemical methods, that after denervation the endplate region of muscle fibres becomes particularly rich in lysosomal enzyme activities. Further studies on the endplate region, using cytochemical staining for lysosomal enzymes at the ultrastructural level may, thus, enable more detailed studies of the lysosomal system in skeletal muscle.

POSSIBLE CLINICAL RELEVANCE OF ENDOCYTOSIS IN SKELETAL MUSCLE

The endocytic activity of muscle fibres affected by various pathological processes has so far received only limited attention. Some observations in the literature suggest, however, that studies of endocytosis in skeletal muscle may lead to a better understanding of the processes operating in certain pathological conditions.

Muscular dystrophies

An increased endocytosis of HRP has been shown to occur in muscles of mice and chickens suffering from hereditary muscular dystrophy (Libelius *et al.* 1978b; 1979b). The uptake occurs in segments of the muscle fibres as it does after denervation, but the distribution of segments has not been studied in dystrophic muscles. In the muscular dystrophies muscle lysosomal enzymes are also increased in activity (see Weinstock and Iodice 1969). The increases in endocytic and lysosomal enzyme activities in dystrophic muscles are not, however, due to denervation since denervation of dystrophic muscles causes further increases in these activities (Libelius *et al.* 1981).

These animal dystrophies are to some extent models of muscular dystrophy in humans. Since similar changes in endocytic and lysosomal enzyme activities are seen in animal models of dystrophic muscle diseases and after denervation, further studies of these phenomena may help to give a better understanding of the pathological processes operating in human dystrophic muscles. For possible future treatment of muscular dystrophy a need for selective administration of drugs to muscles may arise. In such a situation the endocytic process in muscle fibres may be of importance, particularly if it occurs by a receptor-mediated uptake mechanism and is increased in the diseased muscles.

Myasthenia gravis

The turnover of acetylcholine receptors at the neuromuscular junction has been shown to occur by endocytic uptake, degradation in lysosomes and insertion of newly synthesized receptors into the membrane (see Engel and Fumagalli 1982; Fumagalli *et al.* 1982). The endocytic uptake of receptors was shown to be increased in experimental autoimmune myasthenia gravis. Thus, further studies of the different forms of endocytosis in muscle and their relationship to the lysosomal apparatus may also increase our understanding of how acetylcholine receptor levels are regulated in the normal neuromuscular junction, in denervated muscles and in muscles affected by diseases such as myasthenia gravis.

Inflammatory myopathies

Receptor-mediated endocytosis of the kind described in the present work is important not only for the uptake of glycoproteins. Many viruses which infect eucaryotic cells first bind to receptors on the cell surface followed by internalization through endocytosis (see Neville and Chang 1978; Helenius and Marsh 1982). Some viruses, such as Cocksackie A and B viruses, can cause inflammatory myopathies (see Sato and Chou 1978). A characteristic of Cocksackie viruses, however, is that they can infect new-born mice but are by definition unable to infect adult mice. It

has been shown that denervation or poisoning with botulinum toxin renders muscles of adult mice susceptible to infection by Coxsackie A virus (see Sato and Chou 1978; Andrew *et al.* 1984). These treatments also cause increased endocytosis in muscle fibres and this could conceivably be related to the increased susceptibility to infection. Other factors, however, such as other alterations in membrane properties and the possible appearance of virus-specific receptors after denervation may also be of importance.

SUMMARY AND CONCLUSIONS

The uptake of the extracellular markers ^3H -inulin and HRP and the activities of the lysosomal enzymes acid phosphatase, N-acetyl- β -D-glucosaminidase and cathepsin D have been studied in innervated, denervated, botulinum toxin poisoned and reinnervated mouse skeletal muscles, as well as in skeletal muscles from mice treated with the lysosomotropic drug chloroquine. The following conclusions can be drawn from these studies:

1. Skeletal muscle has a significant endocytic activity.
2. The endocytic uptake of HRP in skeletal muscle occurs by a receptor-mediated uptake mechanism based on mannose recognition.
3. Denervation or botulinum toxin poisoning increases the endocytic activity of muscle fibres. The effects of denervation are reversible upon reinnervation.
4. The increase in endocytosis after denervation occurs primarily near the endplate region of muscle fibres.
5. Chloroquine treatment does not influence the *in vivo* uptake of HRP in EDL or tibialis anterior muscles.
6. Denervation or botulinum toxin poisoning increases the activities of lysosomal enzymes in skeletal muscle. The effects of denervation are reversible upon reinnervation.
7. The increases in lysosomal enzyme activities after denervation occur throughout the length of the muscle fibres but are particularly large in the endplate region.

8. Increases in the activities of at least certain lysosomal enzymes may occur without changes in the endocytic activity of skeletal muscle.
9. HRP taken up by denervated muscle fibres accumulates in large phagosomes, at least some of which also participate in autophagic processes, and hence are of a lysosomal nature.
10. The segments of denervated muscle fibres which exhibit high endocytic activity also show ultrastructural signs of myofibrillar degeneration which progresses with time after denervation.
11. The uptake of HRP in denervated muscle fibres occurs in fibre regions which also exhibit a marked proliferation of t-tubuli (from which the HRP uptake is believed to occur), sarcoplasmic reticulum and Golgi complexes.

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APPENDIX

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Membrane and biochemical alterations after denervation and during reinnervation of mouse skeletal muscle

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Denervation of the extensor digitorum longus (EDL) muscle of the mouse by either nerve crush or nerve section produced: a reduction of the resting membrane potential (E_m), alterations in the properties of muscle fibre action potentials and the development of tetrodotoxin (TTX)-resistant action potentials. These changes in membrane electrical properties were accompanied by an increase in the endocytic activity of the muscle and an increase in the activities of the lysosomal enzymes cathepsin D and N-acetylglucosaminidase (NAGA). Reinnervation of muscle was indicated at 9 days after nerve crush by the presence of miniature end-plate potentials. The recovery of membrane electrical properties, beginning with the onset of reinnervation, were not temporally related. The E_m increased in two stages: an early rapid repolarization and a later slower repolarization. The muscle fibers were sensitive to the blocking action of TTX by 12 days after nerve crush, whereas the rate of rise (dV/dt) of the action potential did not approach values of innervated muscles until 21 days. Reinnervation resulted in a decrease in endocytosis and a decrease in the activities of cathepsin D and NAGA toward innervated values by 21 days after nerve crush. The results suggest that membrane alterations after denervation and during reinnervation may occur by endo- and exocytosis of membrane constituents and that the lysosomal system may play a role in the breakdown and/or recycling of these structures.

Key words: Denervation, reinnervation, membrane electrical properties, endocytosis, lysosomal enzymes

Denervation of mammalian skeletal muscle produces alterations in the electrical properties of the sarcolemma. Among these are a fall of the resting membrane potential (E_m), alterations in the muscle fibre action potential and the development of tetrodotoxin (TTX)-resistant action potentials (Thesleff 1962, Redfern & Thesleff 1971*a, b*). The depolarization of the membrane and the development of TTX-resistant action potentials were shown to depend on de novo protein synthesis after denervation, while the alteration in the muscle fibre action potential appeared to be independent of new proteins (Grampp, Harris & Thesleff 1972).

The removal and insertion of membrane proteins may occur by the process of endo- and exocytosis. High rate of endocytosis, as measured by the uptake of extracellular marker molecules, was shown after denervation of skeletal muscle (Libelius et al. 1978). In addition, denervation is reported to result

in increased activities of lysosomal enzymes (Weinstock & Iodice 1969), which may be involved in the break-down and/or recycling of membrane constituents.

The purpose of the present study was to correlate the changes in membrane electrical properties after denervation and during reinnervation with concomitant changes in the rate of endocytosis and the activities of lysosomal enzymes. Some of the results have been presented in a preliminary communication (Sellin et al. 1979).

METHODS

All experiments were conducted in vitro on the extensor digitorum longus (EDL) muscle of adult male NMRI mice. Surgical procedures were done using diethyl ether for anesthesia. Chronic unilateral denervation was performed by removing a 3-4 mm section of the deep peroneal nerve about 10 mm from its entrance into the muscle. For rein-

nervation studies, a 1 mm section of the nerve was crushed in the same position using a fine-tipped forceps. Control muscles were obtained from the contralateral unoperated limbs. At 3, 6, 9, 12, 14, and 21 days after nerve crush or section, the muscles were examined for electrophysiological properties, rate of uptake of macro-molecular tracer or lysosomal enzyme activities.

Electrophysiological properties. At the various times after nerve crush or section the EDL muscles were excised under a continuous flow of oxygenated (95% O₂-5% CO₂) Krebs-Ringer solution having the following composition in mM: NaCl, 135; KCl, 5; CaCl₂, 4; MgCl₂, 1; Na₂HPO₄, 1; NaHCO₃, 15; dextrose, 11. The pH of the solution was 7.2-7.3. The muscles were pinned through their tendons, stretched to approximately their resting lengths, placed in a heated (30±1°C) chamber with a volume of 25 ml and suffused at a rate of 4 ml/min. After a 30 min equilibration period, muscle fibres were examined for resting membrane potentials and action potentials. In addition, miniature end-plate potentials were recorded from some fibres to indicate the presence of reinnervation. For these measurements, glass microelectrodes filled with 3 M KCl and having tip resistances of 10-15×10⁹Ω were used. Amplification and recording of potentials were made using standard techniques (Katz & Thesleff 1957). The procedure for recording action potentials is described in detail elsewhere (Sellin & Thesleff 1979).

Uptake of ³H-inulin. Animals were killed by cervical dislocation at various times following nerve crush or section. The EDL muscles were excised and mounted on perspex holders kept in an oxygenated Krebs-Ringer solution (22°C) as described above, but with 2 mM CaCl₂. Two muscles from each of the two experimental conditions and the four corresponding control muscles were mounted on the same holder. After mounting the muscles were placed in an oxygenated incubation solution at +37°C containing 3.0 µCi/ml of ³H-inulin (The Radiochemical Centre Ltd, Amersham, England) and incubated for 240 min. During the incubation, the solution was continuously bubbled with 95% O₂, 5% CO₂ supplied through a peristaltic pump (flow rate; ca 12 ml/min) to give standardized oxygenation and stirring conditions. After incubation, the muscles were washed in Ringer solution at +4°C for 4 h with the solution being changed each hour. The muscles were then blotted on filter paper, weighed and solubilized in 0.7 ml Soluene-100 (Packard Instrument Company, Inc., Downers Grove Ill., USA) over night at room temperature. Radioactivity was measured by liquid scintillation counting using 10 ml of a toluene-³H-POP mixture. Counting efficiency was calculated from an internal standard with ³H-toluene (Packard Instrument Company Inc., Downers Grove, Ill., USA).

Determination of lysosomal enzyme activities. Cathepsin D activity was measured at pH 4.0 with haemoglobin as the substrate, slightly modified from Barrett (1972). EDL muscles were homogenized in 3 ml ice-cold 0.3 mol/l sucrose in an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) to yield a homogenate of approximately 0.5% muscle tissue (wet weight). The incubation mixture (0.4 ml) contained 0.2 ml muscle homogenate and 2% haemoglobin in a 0.25 mol/l sodium acetate buffer (pH 4.0) with 0.1% Triton X-100. The incubation was

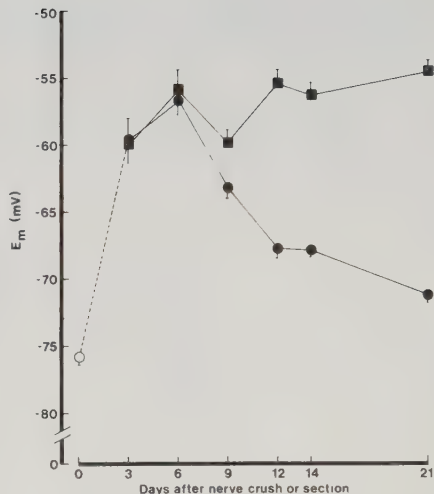


Fig. 1. Resting membrane potential (E_m) (mean $\pm$ S.E., $N=24-206$) in millivolts (mV) recorded from surface fibres in the extensor digitorum longus muscle of the mouse as a function of days after crush (filled circles) or section (squares) of the deep peroneal nerve. The open circle indicates the E_m of normally innervated muscle.

carried out for 120 min at +37°C and stopped by the addition of 2 ml 3% TCA. After centrifugation, 1 ml of the clear supernatant was processed in the Folin-Lowry reaction (Barrett 1972). Appropriate blanks and tyrosine standards were included.

N-acetyl- β -D-glucosaminidase activity was measured after the muscles had been homogenized as described above. Determination of N-acetyl- β -D-glucosaminidase, with 0.1 mmol/l 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside as substrate, was performed in a mixture of 0.075 ml muscle homogenate and 0.5 ml of 0.05 mol/l sodium citrate (pH 4.5) in the presence of 0.1% Triton X-100. The reaction was carried out for 20 min at +37°C and terminated by the addition of 2.5 ml 0.5 mol/l glycine buffer (pH 10.6). The fluorescence was measured in an Aminco Bowman spectrophotofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Appropriate blanks and standards were included.

Protein determinations were performed according to Lowry et al. (1951).

RESULTS

Electrophysiological properties. Denervation of the extensor digitorum longus (EDL) muscle of the mouse produced significant alterations in electrical

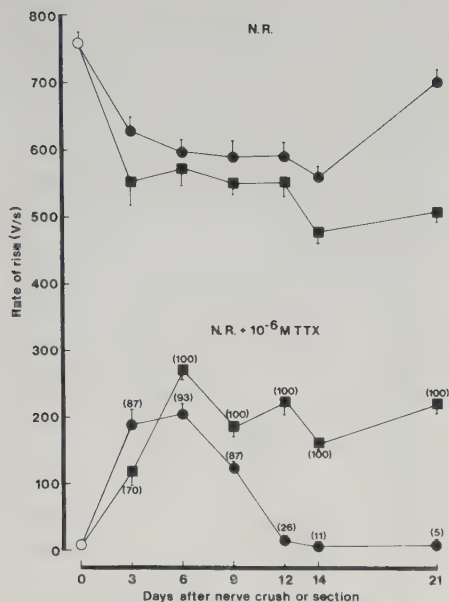


Fig. 2. Rate of rise (mean $\pm$ S.E., $N=24-206$) of the muscle action potential (dV/dt) in volts per second (V/s) recorded from surface fibres in the extensor digitorum longus muscle of the mouse as a function of days after nerve crush (filled circles) or section (squares) of the deep peroneal nerve. The figure is divided into two parameters: the rate of rise in normal Krebs-Ringer solution (N.R.) and in normal Krebs-Ringer solution containing 10^{-6} M tetrodotoxin (N.R. + 10^{-6} M TTX). The figures within parenthesis give the percentage of fibres ($N=31-103$) responding with action potentials in the presence of TTX and the means $\pm$ S.E. are based on these fibres only. The open circles indicate the rate of rise of normally innervated muscles.

properties of the sarcolemma. These changes were similar in magnitude and time-course regardless of whether denervation was accomplished by nerve crush or section. Complete denervation was indicated at 3 and 6 days after surgery by the lack of muscle twitch after nerve stimulation and the absence of miniature end-plate potentials (m.e.p.p.s) from individual muscle fibres. The resting membrane potential (E_m) was reduced from a control (innervated) value of -75.8 ± 0.4 mV to -56.6 ± 1.1 mV and -55.9 ± 1.5 mV at 6 days after nerve crush and section, respectively (Fig. 1). Denervation also produced alterations in some properties of muscle

fibre action potentials (Fig. 2). For example, 6 days following nerve crush or section the rate of rise (dV/dt) of the action potential decreased in both the nerve-crushed (596 ± 15 V/s) and the nerve-sectioned (574 ± 24 V/s) muscles from an initial innervated value of 758 ± 14 V/s. Similarly the threshold potential for action potential generation increased from -41.5 ± 0.9 mV to -47.2 ± 0.9 mV in denervated and to -45.3 ± 0.8 mV in nerve-crushed muscles (9 day values).

At 3 days after denervation, 70–90% of the muscle fibres examined had developed resistance to the blocking action of tetrodotoxin (TTX), and by 6 days all the fibres. The dV/dt of the TTX-resistant action potentials increased to peak values reaching 206 ± 13 V/s (nerve crush) and 273 ± 13 V/s (nerve section) at 6 days after denervation (Fig. 2).

Reinnervation was apparent in the nerve-crushed EDL muscles at 9 days after denervation with the appearance of m.e.p.p.s in individual muscle fibres along with weak muscle twitches after nerve stimulation. M.e.p.p.s and muscle twitches were not observed in any of the nerve-sectioned muscles during the entire time-course of the study. With the onset of reinnervation, there was a slight increase in the E_m for the nerve-crushed muscle (-63.2 ± 0.7 mV) compared to the nerve-sectioned muscle (-59.8 ± 0.9 mV). By the 12th day after nerve crush, the E_m of the reinnervating EDL had increased substantially to -67.7 ± 0.8 mV while the nerve-sectioned muscle remained at -55.3 ± 0.9 mV. After this time the E_m of the reinnervating EDL increased at a slow rate and was near that for normally innervated muscle at 21 days after nerve crush (Fig. 1).

The recovery of the muscle action potential during reinnervation can be divided into two events, the recovery of action potential properties *per se* (e.g. dV/dt) and the recovery of muscle fibre sensitivity to the blocking action of TTX. Between 9 and 12 days after nerve crush, the reinnervating EDL became rapidly sensitive to TTX. This is indicated by the reduction in the number of fibres with TTX resistant action potentials from 87% at 9 days to 26% at day 12 and by a reduction in the dV/dt of the fibres with TTX-resistant spikes (Fig. 2). The dV/dt of the action potential in TTX-containing solution decreased from 206 ± 3 V/s at 6 days after nerve crush to 16 ± 3 V/s at 12 days. In contrast to the TTX-sensitivity, the dV/dt of the action potential in normal Krebs-Ringer solution stayed at values similar to those in denervated fibres at 14 days

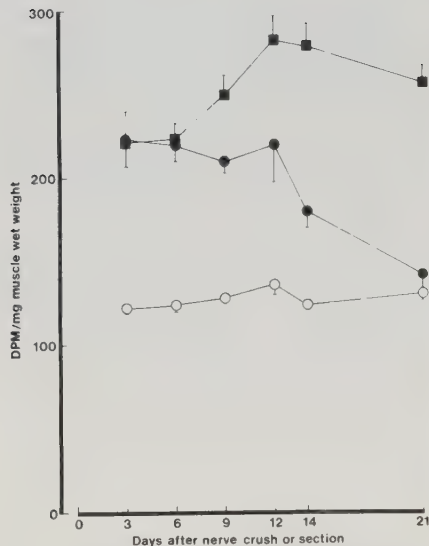


Fig. 3. The uptake (mean $\pm$ S.E.) of ^3H -inulin in disintegrations per minute per milligram muscle wet weight (DPM/mg muscle wet weight) by the extensor digitorum longus muscle of the mouse as a function of days after crush (filled circles; $N=8$) or section (squares; $N=8$) of the deep peroneal nerve. The open circles indicate the uptake of normally innervated muscle ($N=16$).

after nerve crush (562 ± 17 V/s) and did not approach values of innervated muscles (758 ± 14 V/s) until 21 days after nerve crush (705 ± 18 V/s) (Fig. 2). The recoveries towards values of innervated muscles of threshold potential and overshoot of the action potential during reinnervation occurred between 14–21 days.

Uptake of ^3H -inulin. The endocytic activity of EDL muscles after denervation and during reinnervation, as measured by the uptake of ^3H -inulin, is shown in Fig. 3. Denervation by both nerve crush or section produced almost a 100% increase in the uptake per mg wet muscle weight at 3 days after denervation. The nerve-sectioned muscles showed a further increase in uptake between days 6 and 12 followed by a plateau at about 150% of control values by 21 days. In contrast, the nerve-crushed muscles showed no increase between days 6 and 12 and then slowly decreased their uptake after 12

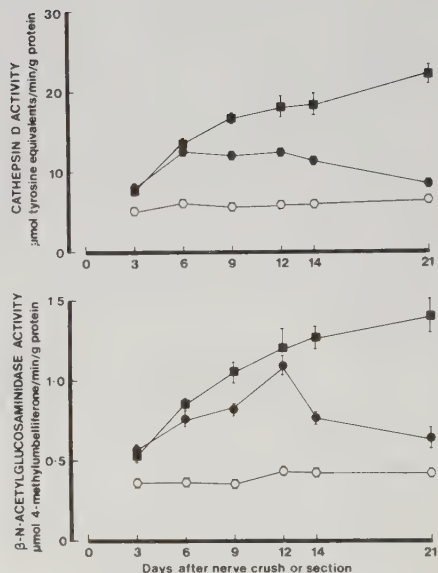


Fig. 4. The activities (mean $\pm$ S.E.) of the lysosomal enzymes cathepsin D (upper panel) expressed as μmol tyrosine equivalents liberated/min/g protein, and N-acetylglucosaminidase (lower panel) expressed as μmol 4-methylumbelliferone liberated/min/g protein. Activities in innervated control muscles (open circles, $N=16$), reinnervating nerve-crushed muscles (filled circles, $N=8$) and chronically denervated muscles (squares, $N=8$) are shown as a function of days after nerve crush or section.

days reaching values similar to normally innervated muscles by 21 days.

Lysosomal enzyme activities. Denervation, by either nerve crush or section, produced similar increases in the activities of the muscle lysosomal enzymes. N-acetylglucosaminidase (NAGA) and cathepsin D (Fig. 4). The activities of these enzymes in the nerve-sectioned muscles increased steadily reaching apparent plateau values at about 21 days. However, the muscles whose nerves had been crushed showed a slower or no increase in the activities between 6 and 12 days and decreased thereafter. By 21 days after nerve crush, the activities of NAGA and cathepsin D were at or near innervated values, while the nerve-sectioned muscles showed 3–4 times higher activities.

DISCUSSION

The fact that some denervation-induced alterations in membrane electrical properties are dependent on newly synthesized proteins (Grampp et al. 1972) suggests that these proteins are incorporated into the muscle membrane. It is likely that this type of membrane turnover may be explained by the process of endo- and exocytosis. The increase in the uptake of ^3H -inulin in denervated skeletal muscle shown in the present study was similar to that reported previously for uptake of extracellular markers in muscle after denervation by the vesiculation and internalization of surface membrane primarily in the t-tubular region (Libelius et al. 1978, Libelius, Josefsson & Lundquist 1979). Since, in the present study there was no change in muscle weight relative to innervated muscle at 3 days after nerve section, the increased uptake per mg muscle clearly indicates an increase in endocytosis. Within that period of time the alterations in membrane electrical properties typical of denervation occurred. The further increase in uptake per mg muscle between 6 and 21 days after nerve section, was complicated by a concomitant loss in muscle weight, which was presumed to have been a decrease in fibre volume rather than number (Pellegrino & Franzini 1963). That is, when the data were plotted as total DPM over muscle (not shown) against days after denervation, the uptake of the nerve-section muscles remained at a high, but constant, level between 3 and 12 days and thereafter displayed an apparent decrease at a slow rate. Therefore, denervation initiated high rates of endocytosis which was maintained until at least 12 days after nerve section. No substantial changes in the membrane electrical properties were observed through 21 days after nerve section beyond the initial denervation-induced alterations, which occurred within the first few days.

For the nerve-crushed muscles, the uptake of ^3H -inulin per mg muscle weight after denervation increased in a fashion similar to the nerve-section muscles. While the uptake per mg muscle increased between 6 and 12 days in the nerve-sectioned muscles, the uptake per mg muscle in the nerve-crushed muscles remained constant during this same period. Since the muscles in both preparations decreased in weight during this time, it may be concluded that the muscles which were reinnervated decreased their endocytic activity. This decrease occurred between

6 and 9 days after nerve crush. At times later than 12 days after nerve crush, the decrease in uptake per mg muscle resulted from the combined effects of a decrease in total uptake per muscle and an increase in muscle weight. It is thus apparent that the increased endocytic activity initiated by denervation is reversed by reinnervation.

Reinnervation occurs between 6 and 9 days after nerve crush because miniature endplate potentials were present at 9 days. The results demonstrated that recovery of some membrane electrical properties during reinnervation were not temporally related. That is, the time-course of recovery differed between individual electrical properties e.g. E_m , rate of rise (dV/dt) of the muscle fibre action potential and TTX-sensitivity. The repolarization of the membrane during reinnervation was similar to that previously reported (McArdle & Albuquerque 1973). The present result suggests that the increase of the E_m occurs in two stages: a rapid increase at the onset of reinnervation between 6 and 12 days, and a slower increase between 12 and 21 days. In addition, dV/dt and TTX-sensitivity did not recover at the same rate as shown previously in a preliminary report (Sellin & McArdle 1977). By 12 days after nerve crush the reinnervated muscles, like innervated muscle were almost completely sensitive to the blocking action of TTX. In contrast the dV/dt in the absence of TTX remained at a value similar to chronically denervated muscle through 14 days after nerve crush and only approached innervated values at 21 days.

The difference in recovery rates between and within the above parameters may be interpreted in the following manner. The changes in membrane electrical properties occurring during reinnervation depend on the morphology and lipid composition of the membrane in addition to the removal and insertion of specific membrane proteins. It appears likely that the early rapid repolarization of the membrane and the early abolishment of TTX-insensitivity are related to the removal of proteinaceous material, responsible for sodium permeability, in the muscle membrane. In regard to E_m , it is believed that the depolarization caused by denervation is, at least in part, secondary to an increase in the sodium leak permeability of the muscle membrane (Robbins 1977). TTX-insensitive channels are presumably voltage sensitive sodium channels. The late slower repolarization could result from conductance changes which, similar to dV/dt , may depend on the

relationship of ion channels to their environment. It is interesting to note that the decrease in membrane resistance, which reflects an increase in ionic conductances, occurs at the later stages of reinnervation after an early increase in E_m (McArdle & Albuquerque 1973).

Earlier studies suggested that increased endocytosis in denervated skeletal muscle may activate lysosomal mechanisms thereby fostering degradative processes (Libelius et al. 1978). The present study provides further evidence for, at least, a temporal relationship between endocytosis and the activity of the lysosomal system. The activities of the proteolytic acid hydrolase cathepsin D and the acid glycosidase N-acetylglucosaminidase (NAGA) increased steadily after nerve section, while levelling off with the onset of reinnervation and then decreased to near innervated values by 21 days after nerve crush in conformity with changes in endocytic activity and membrane electrical properties. The exact mechanisms by which the lysosomal system is implicated in degeneration and recovery of the muscle in denervation-reinnervation remain to be elucidated. It seems likely, however, that the activation of the lysosomal system by endocytosis is responsible for the breakdown and/or recycling of membrane constituents.

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Lysosomes in skeletal muscle following denervation

Time course of horseradish peroxidase uptake and increase of lysosomal enzymes

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Summary. The in-vivo uptake of exogenously applied horseradish peroxidase and the activities of the lysosomal enzymes acid phosphatase and cathepsin D were studied histochemically and/or biochemically in innervated and 2–14 day-denervated tibialis anterior muscles of the mouse. The biochemically determined uptake of horseradish peroxidase showed a large increase already 4 days after denervation. The activities of the lysosomal enzymes increased in a more gradual fashion, and only cathepsin D showed an increase in activity when expressed as total activity per muscle. Histochemically horseradish peroxidase was found to be localized in muscle fibres in characteristic spindle-shaped segments after denervation. The main increase in the number of such segments per transverse section of the muscle occurred between 3 and 6 days after denervation. In serial sections these segments frequently showed positive staining also for acid phosphatase.

It is concluded that exogenously applied horseradish peroxidase is taken up into the lysosomal system, which after denervation becomes organized into characteristic spindle-shaped segments in the muscle fibres. The endocytic activity of muscle fibres increases early after denervation. This is followed by a more gradual increase in activity of lysosomal enzymes and finally by an organization of the lysosomal system into characteristic spindle-shaped segments. The results are compatible with the working hypothesis that increased endocytosis may initiate lysosomal activation in denervated skeletal muscle.

Key words: Acid phosphatase – Cathepsin D – Denervation – Endocytosis – Histochemistry

Denervation of skeletal muscle results in a multitude of changes in surface membrane properties (Thesleff 1974) and increases in the activities of lysosomal enzymes (Weinstock and Iodice 1969; Bird 1975). Another change that occurs after denervation and may be related to the changes in surface-membrane properties is an increased endocytic activity of the muscle fibres, demonstrated by increased uptake of extracellular markers such as horseradish peroxidase (HRP) and ^3H -inulin (Libelius et al. 1978b, 1979b; Sellin et al. 1980; Boegman and Scarth 1981). ^3H -inulin uptake

was shown to obey bulk kinetics (Libelius et al. 1978b); ultrastructural studies (Libelius et al. 1979b) indicated that the uptake of HRP occurred by vesiculation and internalization of membrane primarily in the t-tubular region, and that the HRP then entered lysosomes. A large increase in the incidence of fused pinocytotic vesicles in the extrajunctional membrane of denervated muscle fibres has also been observed using the freeze-fracture technique (Tachikawa and Clementi 1979).

As a working hypothesis it was suggested (Libelius et al. 1978b; Thesleff et al. 1979) that increased endocytosis may initiate lysosomal activation in denervated skeletal muscle.

The main aims of the present investigation were (i) to study with biochemical and histochemical techniques the time course of changes in uptake of HRP and in lysosomal enzyme activities following denervation, and (ii) to compare the distribution, as observed histochemically, of lysosomes and internalized HRP in denervated muscle fibres.

Materials and methods

Animals and preparations

All experiments were performed on the tibialis anterior muscle of adult male NMRI mice, weighing about 30 g. Denervation was carried out by sectioning the deep peroneal nerve just above the knee, under diethyl-ether anaesthesia. Denervation was performed unilaterally, and for the biochemical studies the contralateral muscle was used as control. About half of the animals used were sham operated on the control leg. The animals were sacrificed 2–14 days after denervation by cervical dislocation. Two hours before sacrifice 20 mg of HRP dissolved in 0.2 ml of 0.9% saline was injected into a tail vein. Control animals received saline only.

For biochemical studies the denervated and innervated tibialis anterior muscles were dissected, mounted on perspex holders and washed in four changes of oxygenated Ringer's solution, pH 7.2–7.4 (composition in mM: NaCl, 135; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; Na_2HPO_4 , 1; NaHCO_3 , 15; glucose, 11; bubbled with 95% O_2 –5% CO_2) at 4° C overnight in order to reduce extracellular HRP. Muscles from animals injected only with saline were treated in the same way. After washing, the muscles were blotted dry, weighed, rapidly frozen on a glass dish on crushed dry ice and stored individ-

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ually in sealed plastic tubes at -20°C . The muscles were later homogenized in ice-cold 0.3 M sucrose in an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) to yield a homogenate of 0.5–1.5% muscle tissue (wet weight). The acid phosphatase assay was performed immediately after homogenization and the homogenate was then stored at -20°C until the remaining biochemical assays were carried out.

For histochemical studies muscles were dissected out and pinned onto small pieces of Sylgaard. Cold, buffered formal-calcium fixative (composition: formaldehyde, 4%; CaCl_2 (dried), 10 g/l; tris(hydroxymethyl)aminomethane (Trizma®base), 30 g/l; pH adjusted to 7.2 with HCl or NaOH) was then injected with a 0.4 mm hypodermic needle into the muscle to ensure prompt fixation of all parts. The muscles were then immersed in ice-cold fixative overnight. Following fixation approximately the proximal 1/5 and the distal 1/3 of the muscles were cut away with a razor blade. The remaining pieces were rinsed for 48 h in two changes of an ice-cold solution (composition: sucrose, 7.5%; tris(hydroxymethyl)aminomethane (Trizma®base), 0.1 M; pH adjusted to 7.2 with HCl). The pieces were then oriented and mounted in gum tragacantha on small pieces of cork and frozen in 2-methyl-butane cooled with a mixture of ethanol and dry ice. Sections of 10–14 μm thickness were cut on a cryostat at -18 – 20°C . Muscles were sectioned either longitudinally or transversely with consecutive serial sections collected for HRP or acid phosphatase staining.

For morphometric evaluation of HRP-uptake, muscles were sectioned transversely starting from the proximal end of the muscle pieces. Sixteen sections of 10 μm thickness and separated by 140 μm were taken from each muscle.

Peroxidase (donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7) assay

The muscles were assayed for peroxidase activity according to the method of Lundquist and Josefsson (1971) using a 0.1 M maleic acid-NaOH buffer at pH 6.0 and a glucose oxidase (β -D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) concentration of 1 $\mu\text{g/ml}$ in the reagent solution.

Acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum); EC 3.1.3.2) assay

Acid phosphatase activity was measured at pH 5.0 with 4-methylumbelliferyl-phosphate as the substrate. The incubation mixture contained 50 μl muscle homogenate and 0.5 ml of 50 mM sodium acetate buffer, pH 5.0, containing 0.1 mM 4-methylumbelliferyl-phosphate and 0.01% Triton X-100. The reaction was carried out for 15 min at 37°C and stopped by the addition of 2.5 ml of 0.5 M glycine buffer, pH 10.6. Fluorescence was then measured in an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Appropriate blanks and standards were included.

Cathepsin D (EC 3.4.23.5) assay

Cathepsin D activity was measured at pH 4.0 with haemoglobin as the substrate, slightly modified from Barrett and Heath (1977). The incubation mixture contained 0.2 ml muscle homogenate, 0.2 ml 0.5 M sodium acetate buffer, pH 4.0, and a total of 2% haemoglobin and 0.1% Triton

X-100. The incubation was carried out for 120 min at 37°C and stopped by the addition of 2 ml cold 3% trichloroacetic acid. After centrifugation 0.4 ml of the clear supernatant was diluted to 1.0 ml with 2.5% trichloroacetic acid and then processed in the Folin-Lowry reaction (Barrett and Heath 1977). Appropriate blanks and tyrosine standards were included.

Protein assay

Protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

In order to evaluate changes in HRP-uptake and in lysosomal enzyme activities in the whole muscle and relative to muscle atrophy, activities were calculated both as total activity per muscle and per unit muscle protein.

Histochemical staining for horseradish peroxidase and acid phosphatase

Cryostat sections were collected on coverglasses and air-dried for a few hours. Some sections were stored in a refrigerator overnight without appreciable loss of peroxidase staining.

Staining for HRP was performed by a diaminobenzidine method slightly modified from Graham and Karnovsky (1966). 3,3'-diaminobenzidine tetrahydrochloride (2 mg/ml) was dissolved in 0.2 M phosphate buffer (pH 6.5) and then filtered. H_2O_2 was added to a final concentration of 0.06% and the staining was carried out at room temperature for 30 min. The sections were then washed in saline, dehydrated in ethanol, cleared in xylol and mounted in Pertex.

Staining for acid phosphatase was performed according to Burstone (1958) as modified by Barka (1960) using naphthol AS-BI phosphate as the substrate.

Morphometric evaluation of horseradish peroxidase uptake

The number of HRP-positive fibres in each of 16 transverse sections per muscle was counted in a microscope, by complete visual scanning of each section at a magnification of $\times 160$ – 400 . HRP-positive fibres were defined as fibres containing dense granular HRP-positive inclusions with a characteristic distribution either as a ring in the fibre or centrally located in the fibre. Only rarely were fibres with diffuse HRP-staining encountered and these were not included as HRP-positive fibres.

Statistical evaluation

Statistical significance was tested by means of Student's *t*-test.

Chemicals

Special chemicals used were obtained from the following sources: horseradish peroxidase (type II), tris(hydroxymethyl)aminomethane (Trizma®base) and naphthol AS-BI phosphate from Sigma Chemical Co., St. Louis, Mo., U.S.A.; 4-methylumbelliferyl-phosphate, haemoglobin (crystalline) and 3,3'-diaminobenzidine tetrahydrochloride from British Drug Houses Ltd., Poole, England; glucose oxidase (grade I) from Boehringer, Mannheim, Germany.

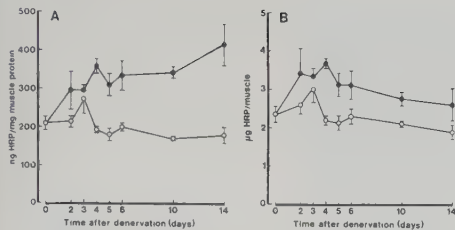


Fig. 1. **A, B.** Peroxidase activity in innervated (open circles) and contralateral 2–14 day-denervated (filled circles) tibialis anterior muscles 2 h after an intravenous injection of 20 mg horseradish peroxidase. Endogenous peroxidase activity in the muscles was only 2–3% of the total activity and has not been subtracted from the values shown. **A** Peroxidase activity expressed as ng horseradish peroxidase/mg muscle protein. **B** Peroxidase activity expressed as µg horseradish peroxidase/muscle. Mean values $\pm$ S.E.M. ($n=6$ for all points except the 0 day value where $n=12$). The difference between denervated and innervated muscles was statistically significant ($p<0.05$) from 4 days after denervation and onwards except for the difference at 14 days in B

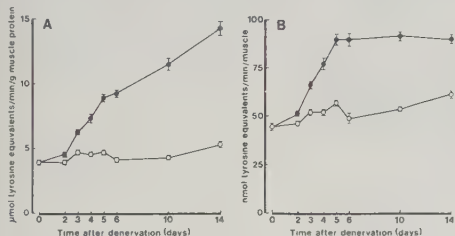


Fig. 2. **A, B.** Cathepsin D activity in innervated (open circles) and contralateral 2–14 day-denervated (filled circles) tibialis anterior muscles. **A** Cathepsin D activity expressed as µmol tyrosine equivalents liberated/min/g muscle protein. **B** Cathepsin D activity expressed as nmol tyrosine equivalents liberated/min/muscle. Mean values $\pm$ S.E.M. ($n=12$ for all points except the 0 day value where $n=24$)

Results

Time-course of atrophy

Following the second day after denervation muscle weight and total protein content decreased almost linearly up to 14 days after denervation. By the 14th day after denervation muscles had lost about 45% in both weight and total protein content as compared to the corresponding innervated controls. During the 14-day experimental period innervated control muscles increased slightly, but not more than 15%, in both weight and total protein content.

Biochemical assay of horseradish peroxidase uptake

The uptake of HRP recorded as HRP-content/mg muscle protein increased to about 185% of the innervated control value by 4 days after denervation (Fig. 1A). The content of HRP/mg muscle protein then remained at a fairly constant level showing a slight further increase at 14 days after denervation. The content of HRP/muscle (Fig. 1B) also showed a peak increase at 4 days after denervation (about

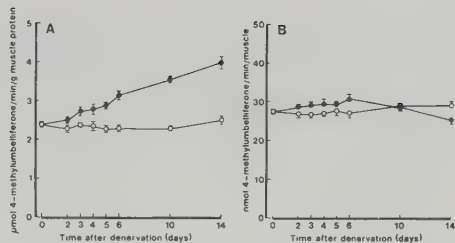


Fig. 3. **A, B.** Acid phosphatase activity in innervated (open circles) and contralateral 2–14 day-denervated (filled circles) tibialis anterior muscles. **A** Acid phosphatase activity expressed as µmol 4-methylumbelliferone/min/g muscle protein. **B** Acid phosphatase activity expressed as nmol 4-methylumbelliferone/min/muscle. Mean values $\pm$ S.E.M. ($n=12$ for all points except the 0 day value where $n=24$)

170% of the corresponding innervated controls). The content of HRP/muscle then decreased again but remained higher than the innervated controls although at 14 days after denervation this difference was not statistically significant.

The endogenous peroxidase activity represented the equivalent of 4.7 ± 0.2 ng HRP/mg protein ($n=18$) in innervated muscles and 7.9 ± 0.3 ng HRP/mg protein ($n=6$) in 14 days denervated muscles. This represents no more than 2.3% of the total peroxidase activity in muscles from animals injected with HRP. Values for HRP-uptake have therefore not been corrected for endogenous peroxidase activity.

Lysosomal enzyme activities

The activities of cathepsin D expressed per g muscle protein and per muscle at various times after denervation are shown in Fig. 2A, B. The activity/g muscle protein (Fig. 2A) increased in two phases after denervation. Initially there was a rapid increase occurring between days 2 and 5, and subsequently there was a slower increase up to 14 days after denervation. The activity of cathepsin D/muscle showed an increase of about 75% occurring between days 2 and 5 after denervation. The activity then remained fairly constant up to 14 days after denervation.

Corresponding values for acid phosphatase activities are shown in Fig. 3A, B. The activity/g muscle protein (Fig. 3A) increased gradually starting at the second to third day after denervation. The acid phosphatase activity expressed as total activity/muscle (Fig. 3B) did not show any distinct difference between denervated and innervated muscles.

Horseradish peroxidase and acid phosphatase histochemistry

In muscles denervated more than 3–4 days circumscribed bodies with granular appearance containing peroxidase activity could be seen with a characteristic distribution in the muscle fibres. In longitudinal sections the HRP-positive bodies were distributed in a spindle-shaped fashion leaving the most peripheral and central regions of the fibre devoid of peroxidase activity. At the ends of such a segment the HRP-positive bodies became more centrally located, frequently smaller in size, and gradually faded away (Fig. 4A,

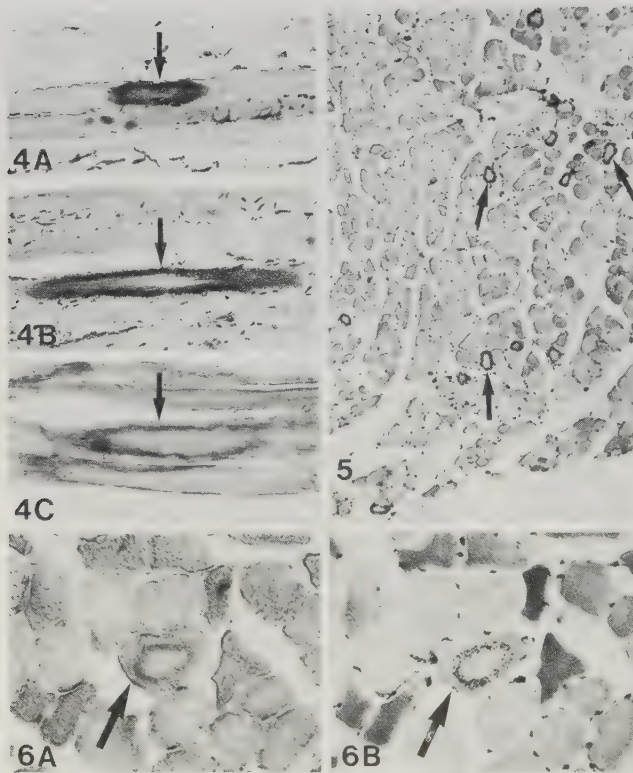


Fig. 4A, B. Longitudinal sections of 14-day-denervated tibialis anterior muscle 2 h after an intravenous injection of horseradish peroxidase. Horseradish peroxidase uptake (arrows, dark brown colour in preparation) is seen in muscle fibre segments of varying length. **C** Longitudinal section of 14-day-denervated tibialis anterior muscle. Acid phosphatase staining (arrow, red colour in preparation) is seen with a distribution similar to that for horseradish peroxidase in **A** and **B**. **A** $\times 145$, **B** $\times 185$, **C** $\times 200$

Fig. 5. Transverse section of 14-day-denervated tibialis anterior muscle 2 h after an intravenous injection of horseradish peroxidase. Numerous fibres (some denoted by arrows) show HRP-uptake with a ring-like distribution. $\times 95$

Fig. 6A, B. Consecutive transverse sections of 14-day-denervated tibialis anterior muscle 2 h after an intravenous injection of horseradish peroxidase. Arrows denote a fibre stained for both acid phosphatase (**A**) and horseradish peroxidase (**B**). **A** and **B** $\times 280$

B. The length of individual segments varied considerably from about 100 to 600 μm . In transverse sections the HRP-positive bodies were usually seen distributed in a ring or occasionally centrally in the fibre depending on at which level of the segment the fibre had been cut. In some sections, especially from muscles taken longer times after denervation (6–14 days), up to 35–40 HRP-positive fibres could be seen in one section (Fig. 5). In denervated muscles from animals not injected with HRP no peroxidase-positive structures were observed.

Staining for acid phosphatase could be seen with a similar distribution in the muscle fibre as that described above for HRP (Fig. 4C). Frequently, positive staining for HRP coincided with positive staining for acid phosphatase in consecutive serial sections (Fig. 6A, B). However, the intensity of acid phosphatase staining varied somewhat probably due to varying enzyme inactivation by fixation. Acid phosphatase staining with a distribution similar to that described above for HRP was sometimes observed without corresponding HRP-staining in serial sections and could also be seen in denervated muscles from animals not injected with HRP. Staining for acid phosphatase activity was negative if the sections were pretreated with 10 mM NaF, an inhibitor of acid phosphatase, and when NaF was included

in the staining medium. The HRP and acid phosphatase stainings as described above for denervated muscles were never observed in normal innervated muscle.

Morphometric evaluation of horseradish peroxidase uptake

The average number of HRP-positive fibres per transverse section calculated from 16 sections from each of 2–10 muscles taken at various times after denervation is shown in Fig. 7. As shown in the figure the main increase in the number of HRP-positive fibres occurred between 3 and 6 days after denervation.

HRP-positive segments did not seem to be evenly distributed throughout the denervated muscle. In Fig. 8, which shows the pooled data from 39 muscles taken 3 to 14 days after denervation, it can be seen that more proximal sections contained a higher proportion of the total number of positive fibres counted per muscle than did more distal sections. Since all muscles were dissected and cut in a similar manner, this variation in distribution will not greatly influence the values shown in Fig. 7, but indicates a preferential occurrence of HRP-positive segments in more proximal parts of the muscle.

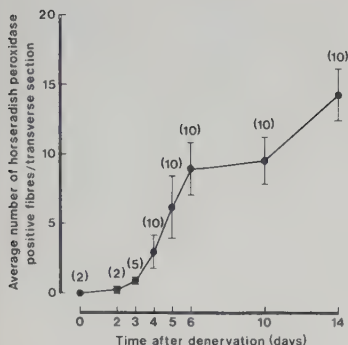


Fig. 7. Average number of horseradish peroxidase-positive fibres (as defined in Materials and methods)/transverse section of innervated and 2–14 day-denervated tibialis anterior muscles 2 h after an intravenous injection of horseradish peroxidase. Sixteen sections of 10 μ m thickness and separated by 140 μ m were cut from each muscle and the number of horseradish peroxidase-positive fibres in each section counted. From innervated and 2-day-denervated muscles 8 sections per muscle were counted. Mean $\pm$ S.E.M. Number of muscles counted at each time point after denervation given in brackets

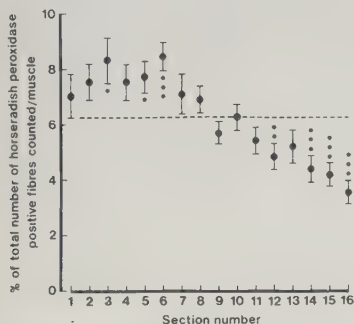


Fig. 8. Distribution of horseradish peroxidase-positive fibre segments in denervated tibialis anterior muscle 2 h after an intravenous injection of horseradish peroxidase. The percentage in each section of the total number of horseradish peroxidase-positive fibres counted in the muscle is given for 16 sections taken from different levels of the muscles. Muscles were treated as described in Fig. 7. Section number 1 corresponds to the most proximal section and section number 16 to the most distal section. The dashed horizontal line shows the expected percentage if the horseradish peroxidase-positive fibre segments were evenly distributed in the muscle. Mean $\pm$ S.E.M. ($n=39$ muscles taken 3–14 days after denervation). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Levels of significance from the dashed line

Discussion

Uptake of horseradish peroxidase

The present study shows that the tibialis anterior muscle responds in vivo to denervation by increased uptake of the extracellular marker HRP. This confirms the results of previous studies where an increased uptake of HRP in denervated muscle was observed in vivo (Libelius et al. 1979b) and in vitro (Libelius et al. 1978b).

The uptake of HRP, whether expressed as total HRP-content/muscle or expressed as HRP-content/mg muscle protein, was found to be maximal or near maximal already 4 days after denervation. Further changes in HRP-uptake, at later times after denervation, were more gradual and may be influenced by the muscle fibre atrophy and intracellular processing of HRP, which is so far poorly understood.

Lysosomal enzyme activities

Many previous studies have shown that denervation causes increased activities of muscle lysosomal enzymes (see above). In the present study the activities of cathepsin D and acid phosphatase were examined at various times after denervation. When activities were expressed/g muscle protein both enzymes showed increased activities after denervation, the increases becoming apparent at 2–3 days after denervation. For cathepsin D there was also a marked increase in activity after denervation when the activity was expressed as total enzyme activity/muscle. This indicates an increased rate of synthesis and/or a reduced rate of degradation of this enzyme following denervation. For acid phosphatase, however, there was no distinct difference between denervated muscles and innervated controls when activity was expressed as total enzyme activity/muscle. This would seem to indicate no relative change in rates of synthesis and degradation of this enzyme following denervation although the enzyme is spared from the general protein degradation that occurs in the denervated muscle. However, some acid phosphatase activity may derive from extralysosomal sources (for discussion, see Weinstock and Iodice 1969; Meijer et al. 1979, 1980) and the possibility that the observed constant activity of acid phosphatase/muscle could be the result of an increase in lysosomal activity, and a concomitant decrease in extralysosomal activity can therefore not be excluded. Histochemical evidence (see below) showing the appearance of acid phosphatase-positive structures in denervated muscle fibres, however, indicates a local increase in lysosomal acid phosphatase activity and/or a redistribution of existing enzyme to characteristic spindle-shaped segments of the muscle fibres.

Horseradish peroxidase and acid phosphatase histochemistry

The biochemically measurable HRP-uptake and acid phosphatase activity in innervated muscle was not corroborated by histochemical means. The reason for this is not clear but may be due to the HRP-uptake and acid phosphatase activity occurring diffusely in the fibres, giving local enzyme activities below the limit of histochemical detection. Part of the HRP-uptake and acid phosphatase activity may also derive from other cellular elements than muscle fibres, although it has been suggested (Canonica and Bird 1970) that only a small part of muscle lysosomal enzymes, such as acid phosphatase and cathepsin D, derives from macrophages and other connective tissue cells.

After denervation acid phosphatase activity could be demonstrated histochemically in characteristic spindle-shaped segments of muscle fibres. Some loss of acid phosphatase activity probably occurred as a result of fixation, and this may explain the sometimes rather faint staining for acid phosphatase.

Following denervation HRP could also be found localized segmentally in muscle fibres in structures similar to the above-described containing acid phosphatase activity, and in serial sections the same spindle-shaped segments could frequently be seen to stain positively for both acid phosphatase and HRP. It is therefore considered likely that after denervation HRP accumulates in muscle fibre lysosomes, which have become organized into the described spindle-shaped segments. This is in agreement with previous ultrastructural findings (Libelius et al. 1979b) indicating that HRP accumulates in heterophagosomes and lysosomes. Since HRP-staining was more reliable than acid phosphatase staining, the appearance of HRP-positive fibres (as defined in Materials and Methods) was used for studying the time course for the organization of muscle fibre lysosomes. Fibres with the described distribution of acid phosphatase activity were sometimes encountered without positive HRP-staining in corresponding serial sections. Thus, it would seem that some lysosomes did not accumulate HRP and therefore the number of HRP-positive segments may not be an accurate estimate of the number of lysosome segments in the muscle fibres.

The localized acid phosphatase activity and HRP-uptake in segments of muscle fibres were not observed in sections of fresh-frozen denervated muscle. This may be due to a freezing-induced diffuse dispersion of the enzyme into the unfixed tissue or an extraction of the enzyme during staining (see also Barka and Anderson 1962). However, in fixed denervated muscles acid phosphatase activity could be seen with the described ring distribution in transverse sections also in muscles from animals not injected with HRP. Therefore, the characteristic distribution of lysosomes is a result of denervation and independent of HRP-administration.

The HRP-positive segments occurring in the denervated muscle fibres were not evenly distributed throughout the muscle. In the 2.4 mm of the muscles that were sectioned for morphometric evaluation of HRP-uptake there was a significant decrease in the number of HRP-positive fibres/section from proximal to more distal sections. The explanation for this uneven distribution of the HRP-uptake must await further experimentation.

Time course of changes in endocytic activity and in the lysosomal apparatus following denervation

One of the aims of the present study was to investigate and compare the time course of changes in endocytic activity, lysosomal enzyme activity and organization of the lysosomal system in skeletal muscle after denervation. In order to make such a comparison, the increases that occurred in denervated muscles, as compared to innervated controls, in the different parameters (uptake of HRP/mg muscle protein (Fig. 1A), cathepsin D activity/g muscle protein (Fig. 2A) and HRP-positive fibres/transverse section (Fig. 7)) at 14 days after denervation may be set to 100%. The fraction of this increase that has occurred in the different parameters may then be compared at the different time points studied. This analysis is shown in synoptic form in Fig. 9A, and shows that the endocytic activity responds with an almost step-like increase occurring between days 3 and 4 after denervation. This is followed by a more gradual increase in the activity of the lysosomal enzyme cathepsin D, and finally by the organization of the lysosomal sys-

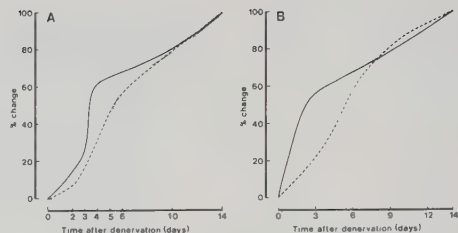


Fig. 9A, B. Time course of changes in endocytic activity (—), cathepsin D activity (---) and organization of the lysosomal system (....) in skeletal muscle after denervation. The increases that occurred in 14 day-denervated muscles as compared to innervated controls have been set to 100%. The mean fractions of this increase that occurred at the different time points studied are shown in stylized form. A. Data from the present study (taken from Figs. 1A, 2A, 7). B. Data from Sellin et al. (1980) (Figs. 3, 4)

tem. A similar analysis of the data obtained by Sellin et al. (1980) for the in-vitro uptake of ^3H -inulin in mouse extensor digitorum longus muscle and the activity of cathepsin D in the same muscle after denervation is shown in Fig. 9B. The figure similarly illustrates an initial increase in endocytic activity followed by an increase in the activity of cathepsin D.

The working hypothesis that increased endocytosis may initiate lysosomal activation (Libelius et al. 1978b; Thesleff et al. 1979) in denervated muscle is compatible with the results presented here. The present study also shows that the lysosomal system in skeletal muscle responds to denervation not only by an increase in the activities of lysosomal enzymes but also by a reorganization of the lysosomal apparatus into characteristic spindle-shaped segments in the muscle fibre. The fibre segments showing HRP-uptake described in the present study are similar to those earlier observed in denervated mouse muscle (Libelius et al. 1979b) and in dystrophic mouse and chicken muscle (Libelius et al. 1978a, 1979a). A common denominator for denervated and dystrophic muscle is a change in surface membrane properties (Thesleff 1974; Rowland 1980), and it is conceivable that these changes in membrane properties may be accompanied by an increase in endocytic activity.

Several interesting questions related to the present study remain without definite answers, for example, why the lysosomes become organized in a spindle-like fashion, whether all muscle fibres are affected at one time, and whether the lysosomes are related to muscle fibre atrophy or degenerative changes.

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Uptake of Horseradish Peroxidase in Denervated Skeletal Muscle Occurs Primarily at the Endplate Region

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SUMMARY

The spatial distribution of horseradish peroxidase (HRP) uptake has been studied by light- and electron microscopy in the denervated hemidiaphragm of the mouse. Segments with high HRP uptake were observed in a band centrally located in the denervated muscle. This distribution is similar to the well-known innervation pattern of the diaphragm. Ultrastructural studies demonstrated a high incidence of post-synaptic folds in close proximity of fibre areas with high intracellular content of HRP. 8–12 days after denervation a large number of fibres showed segments of high HRP uptake. 2–4 days after denervation very few such segments were observed. Biochemical studies also demonstrated an increase in HRP uptake after denervation occurring primarily in the endplate region. The activities of the lysosomal enzymes *N*-acetyl- β -D-glucosaminidase, acid phosphatase and cathepsin D all increased after denervation, most prominently in the endplate region.

It is suggested that the observed segmental uptake of HRP and lysosomal activation reflects a process for rapid membrane turnover in denervated muscle.

Key words: *Acid phosphatase – Cathepsin D – Denervation – Endocytosis – Endplate – Horseradish peroxidase – Lysosomal enzymes – N-acetylglucosaminidase – Skeletal muscle*

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INTRODUCTION

Increased endocytosis of extracellular markers in denervated muscle has been demonstrated with radiochemical, biochemical, histochemical and ultrastructural methods (Libelius et al. 1978; 1979; Tågerud and Libelius 1984). The histochemical and ultrastructural studies have shown that the uptake of horseradish peroxidase (HRP) in denervated muscle occurs in characteristic spindle-shaped segments of the muscle fibres. These segments also show positive staining for the lysosomal enzyme acid phosphatase (Tågerud and Libelius 1984). In a recent study of the denervated tibialis anterior muscle of the mouse the segments with HRP were found to be unevenly distributed, with uptake of HRP occurring preferentially in a limited area of the muscle (Tågerud and Libelius 1984). Ultrastructural studies indicated a prevalence of post-synaptic folds in the vicinity of segments with HRP uptake (unpublished observations).

The aim of the present investigation was to study, in more detail, the distribution of segments of high HRP uptake in denervated muscle and its possible spatial relationship to the endplate region. The denervated mouse hemidiaphragm was chosen since it is a thin and wide muscle with a well defined innervation pattern.

MATERIALS AND METHODS

All experiments were performed on adult male NMRI mice weighing about 30 g. Denervation of the left hemidiaphragm was carried out under diethyl-ether anesthesia as follows: A small incision was made through the skin and the intercostal muscles. A glass hook was inserted into the thoracic cavity and the left phrenic nerve was hooked up, where it passes from the heart to the diaphragm, pulled out and sectioned. With this method of denervation no reinnervation occurs within the time period studied (see Smith and Thesleff 1976).

Animals were killed 2–12 days after denervation by cervical dislocation. 2 h before killing 20 mg of HRP dissolved in 0.2 ml of 0.9% saline was injected into a tail vein.

After dissection, the diaphragm was pinned onto a frame of Sylgaard resin. Muscles to be used for gross morphological or biochemical studies were washed in four changes of oxygenated Ringer's solution, pH 7.2–7.4 (composition in mM: NaCl, 135; KCl, 5; CaCl₂, 2; MgCl₂, 1; Na₂HPO₄, 1; NaHCO₃, 15; glucose, 11; bubbled with 95% O₂–5% CO₂) at 4 °C overnight to reduce extracellular HRP.

Gross morphological studies

Muscles were fixed overnight in an ice-cold buffered formol-calcium fixative (composition: formaldehyde, 4%; CaCl₂ (dried), 10 g/l; tris (hydroxymethyl) amino-methane (Trizma[®] base), 30 g/l; pH adjusted to 7.2 with HCl or NaOH). Following fixation muscles were rinsed for at least 48 h in two changes of an ice-cold wash solution (composition: sucrose, 7.5%; Trizma[®] base, 0.1 M; pH adjusted to 7.2 with HCl). Staining for HRP was performed by a diaminobenzidine method utilizing glucose oxidase and D-glucose for H₂O₂ production. 3,3'-diaminobenzidine hydrochloride

(0.1 mg/ml), glucose oxidase (1 μ g/ml) and D-glucose (2 mg/ml) were dissolved in the wash solution and staining was carried out for 3–4 h at room temperature.

Ultrastructural studies

Muscles were fixed for 2 h in an ice-cold fixative (composition: glutaraldehyde, 2%; paraformaldehyde, 1%; CaCl_2 ($2\text{H}_2\text{O}$), 20 mg/l; phosphate buffer, pH 7.3, 0.12 M). After fixation the muscles were rinsed in an ice-cold wash solution (composition: sucrose, 7.5%; CaCl_2 ($2\text{H}_2\text{O}$), 20 mg/l; phosphate buffer, pH 7.3, 0.12 M). Muscles were then stained for peroxidase activity by incubating for 3–4 h at 4 °C in wash solution containing 3,3'-diaminobenzidine hydrochloride (1 mg/ml), glucose oxidase (0.2 mg/ml) and D-glucose (2 mg/ml). Fibres with segments of high peroxidase activity were then dissected from the muscles, postfixed in 2% OsO_4 in 0.1 M phosphate buffer for 2 h at room temperature, rinsed in 2.4% NaCl, dehydrated and embedded in Polarbed 812. Semithin sections (1–2 μ m) were inspected in the light microscope. Ultrathin sections from selected areas were stained with lead citrate and uranyl acetate and examined in a Jeol CX 100 electron microscope.

Biochemical studies

Regions considered to contain a majority of endplates (endplate regions) and regions devoid of endplates (non-endplate regions) were cut out from the innervated and denervated hemidiaphragms. The muscle pieces obtained were then blotted dry, weighed, rapidly frozen on a glass dish on crushed dry ice and stored individually in sealed plastic tubes at –20 °C. Each muscle piece was later homogenized in 2 ml ice-cold 0.3 M sucrose in an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, F. R. G.). The muscle homogenates were assayed for peroxidase, *N*-acetyl- β -D-glucosaminidase, acid phosphatase and cathepsin D activities as reported previously (Sellin et al. 1980; Tägerud and Libelius 1984).

Protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

Statistical evaluation

Statistical significance was tested by means of Student's *t*-test using paired observations.

Chemicals

Special chemicals used were obtained from the following sources: horseradish peroxidase (type II) and tris(hydroxymethyl)aminomethane (Trizma[®] base) from Sigma Chemical Co., St. Louis, MO, U.S.A.; 3,3'-diaminobenzidine tetrahydrochloride, haemoglobin (crystalline) and 4-methyl-umbelliferyl-phosphate from British Drug Houses Ltd., Poole, U.K.; glucose oxidase (grade I) from Boehringer, Mannheim, F. R. G.; glutaraldehyde (8% aqueous, E. M. grade) from Polysciences Inc., Warrington, PA, U.S.A.; 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide from Serva Feinbiochemica, Heidelberg, F.R.G.

RESULTS

Muscles were examined morphologically for HRP uptake 2, 4 and 8–12 days after denervation. For the gross morphological studies background staining was found to be reduced if low concentrations of diaminobenzidine and glucose oxidase were used and if formol–calcium fixative was used instead of the glutaraldehyde/paraformaldehyde fixative.

Gross morphological studies

In muscles denervated for 8–12 days peroxidase staining could be seen in the denervated half of the diaphragm by visual inspection with the unaided eye or by examination with a dissecting microscope (Figs. 1 and 2). The peroxidase staining occurred segmentally in the muscle fibres, the segments being distributed in a band centrally located in the muscle fibres (Figs. 1 and 2). In muscles denervated for 2 days no peroxidase-positive segments were observed and in muscles denervated for 4 days only occasional segments of peroxidase activity were observed. No segmental peroxidase staining was observed in the innervated half of the diaphragm (Fig. 1) or in denervated muscles not injected with HRP.

Ultrastructural studies

Only superficial fibres in the diaphragm showed acceptable peroxidase staining, probably because of poor penetration of the staining medium into the muscle.

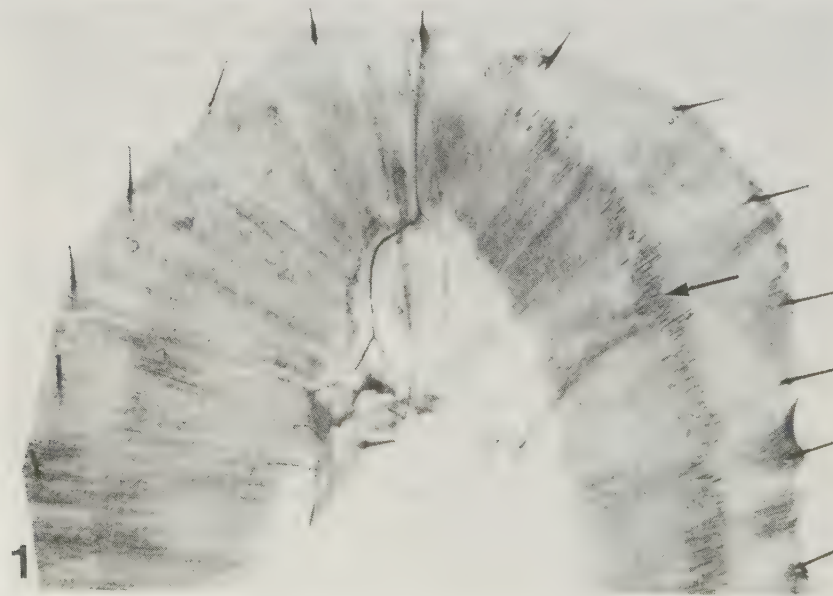


Fig. 1. 12-days denervated mouse hemidiaphragm 2 h after an intravenous injection of HRP. Note the central localization of HRP segments (arrow) in the denervated half of the diaphragm. $\times 5$.

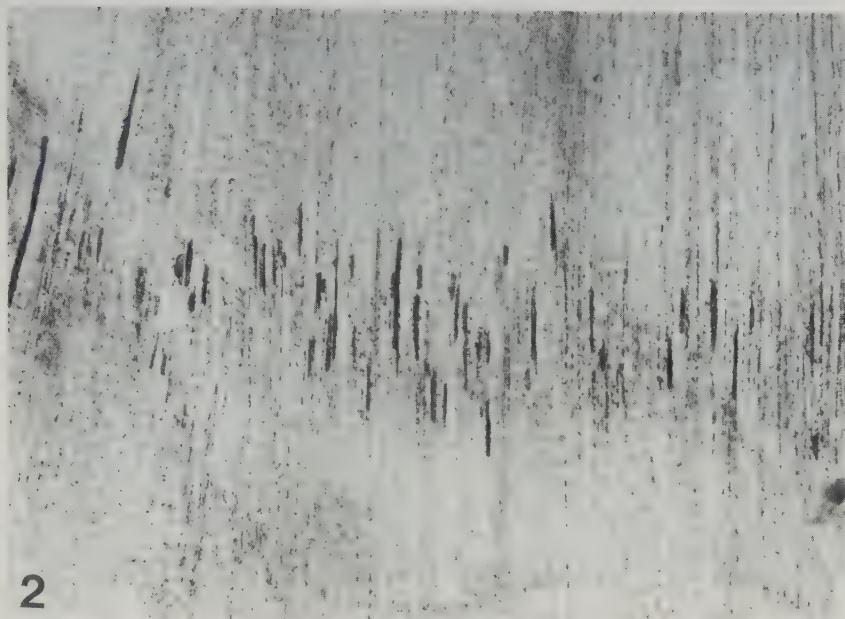


Fig. 2. A higher magnification of the same preparation as in Fig. 1. $\times 27$.

Figs. 3 and 4 demonstrate the electron microscopic appearance of a denervated muscle fibre with HRP uptake in the endplate region. The central myofibrillar degeneration which can be seen in Fig. 3 has been a rather frequent observation in fibre segments with high HRP uptake (unpublished observations). Post-synaptic folds or remnants thereof were observed in the vicinity of the area with high intracellular HRP content in a fair number of the fibres examined in the electron microscope.

Biochemical studies

The biochemical studies were performed on innervated and 10-days denervated hemidiaphragms.

The uptake of HRP in the endplate regions and non-endplate regions of innervated and denervated hemidiaphragms is shown in Fig. 5. The HRP content/g muscle protein increased in the denervated endplate regions by about 45% compared to the corresponding innervated endplate regions. Outside the endplate regions there was no significant change in HRP uptake after denervation.

The endogenous peroxidase activity in the muscle pieces represented less than 2% of the total peroxidase activity in muscle pieces from animals injected with HRP. Values for HRP uptake have not been corrected for this endogenous peroxidase activity.

Fig. 6 shows the activities of the lysosomal enzymes *N*-acetyl- β -D-glucosaminidase (Fig. 6A), acid phosphatase (Fig. 6B) and cathepsin D (Fig. 6C) in the endplate regions and non-endplate regions of innervated and denervated hemidiaphragms. The

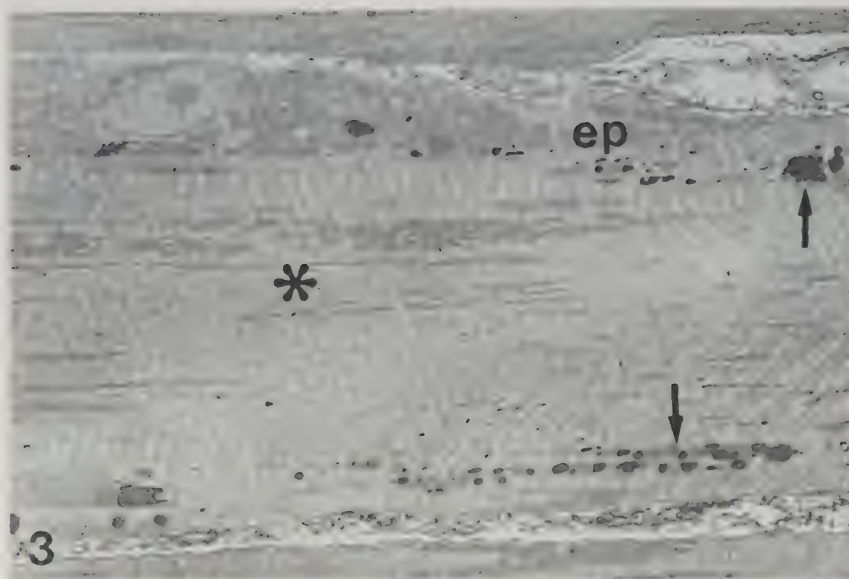


Fig 3. Electron micrograph of a 11-days denervated muscle fibre showing the endplate region (ep) and HRP-positive bodies (arrows). Note the central myofibrillar degeneration with loss of cross-striation (asterisk). $\times 2350$.

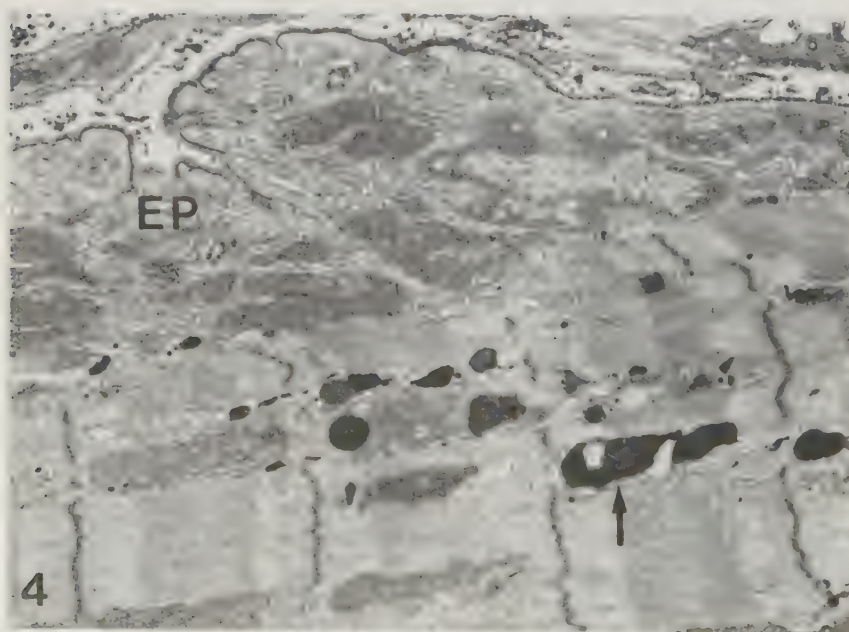


Fig 4. A higher magnification of the same specimen as in Fig 3 illustrating HRP-positive bodies (one denoted by arrow) located near the endplate region (EP). $\times 11750$.

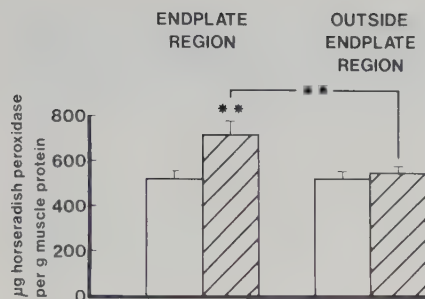


Fig. 5. Peroxidase activity, expressed as μg horseradish peroxidase/g muscle protein, in endplate and non-endplate regions of innervated (□) and 10-days denervated (▨) hemidiaphragms 2 h after an intravenous injection of 20 mg horseradish peroxidase. Mean values $\pm$ SEM ($n = 19$). ** $P < 0.01$.

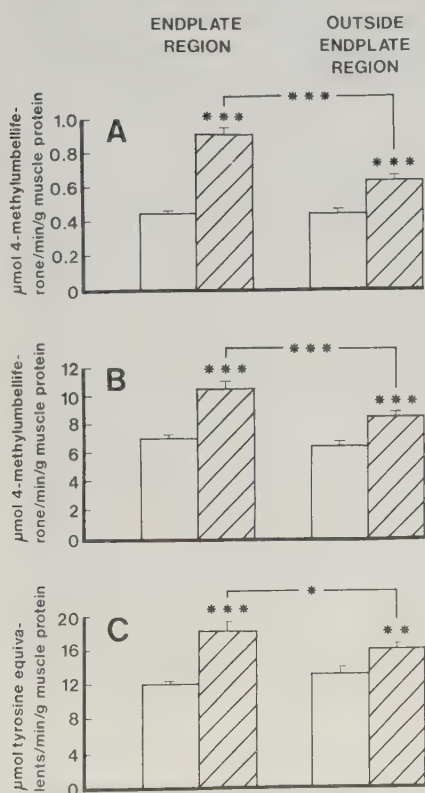


Fig. 6. Lysosomal enzyme activities in endplate and non-endplate regions of innervated (□) and 10-days denervated (▨) hemidiaphragms. *A*: *N*-acetyl-β-D-glucosaminidase activity expressed as μmol 4-methylumbelliferone liberated/min/g muscle protein. *B*: Acid phosphatase activity expressed as μmol 4-methylumbelliferone liberated/min/g muscle protein. *C*: Cathepsin D activity expressed as μmol tyrosine equivalents liberated/min/g muscle protein. Mean values $\pm$ SEM ($n = 21$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

activities of these enzymes per g muscle protein increased by about 52–109% in the denervated endplate regions as compared to the corresponding innervated endplate regions. Outside the endplate regions the corresponding increases were about 28–47%.

DISCUSSION

The results presented here indicate that endocytic activity in denervated muscle fibres occurs mainly in the vicinity of the endplate region. This is suggested by the distribution of HRP segments in the denervated hemidiaphragm, which co-incides with the characteristic innervation pattern of the muscle (Waser and Lüthi 1956). The ultrastructural studies demonstrating a high incidence of post-synaptic folds in close proximity to HRP segments give further support to this concept. The biochemical studies also indicate an increase in HRP uptake primarily in the endplate region after denervation. The activities of the three lysosomal enzymes *N*-acetyl- β -D-glucosaminidase, acid phosphatase and cathepsin D were found to be increased both in the endplate region and outside the endplate region of the denervated muscle fibres. For all enzymes, however, the increase was found to be most prominent in the central part of the muscle fibres (the endplate region, Fig. 6A–C).

Following denervation there is a rapid turnover of muscle surface-membrane proteins (see Thesleff 1974; Fambrough 1979). Degradation is believed to occur in lysosomes of the muscle fibres (see Fambrough et al. 1977). The segments of denervated muscle fibres showing high uptake of HRP have previously been shown also to contain high activity of the lysosomal enzyme acid phosphatase (Tågerud and Libelius 1984). This, together with the results presented here, suggests that, after denervation, it is mainly the central part of the muscle fibre (the endplate region) that becomes an active site where uptake of membrane and membrane components occurs, followed by degradation in lysosomes. It is not clear whether the increased surface membrane area of the endplate region (due to post-synaptic folding) has any relevance to the described phenomena. However, HRP uptake structures have not been observed to originate from the post-synaptic membrane but rather seem to be derived from transverse tubules (Libelius et al. 1979, and unpublished observations).

The HRP uptake in skeletal muscle has recently been found to occur by a receptor-mediated process where the binding sites most likely recognize mannose residues on the HRP molecule (Tågerud and Libelius, in preparation). However, acid phosphatase positive segments, similar to those described above containing HRP, are present also in denervated muscles from animals which did not receive HRP (Tågerud and Libelius 1984) showing that HRP is not a prerequisite for the described focal changes in the denervated muscle fibres.

Previous ultrastructural studies have indicated that the segmental uptake of HRP in denervated skeletal muscle occurs by vesiculation and internalization of membrane, primarily from the t-tubules (Libelius et al. 1979). Another alteration which has been described in denervated muscle and which originates from t-tubules is the appearance of irregularly discharging fibrillation potentials (see Thesleff 1982). These potentials are particularly prevalent in the denervated mouse diaphragm muscle where they reach a

maximum 9–12 days after denervation. The potentials start in the central part of the muscle fibres and are presumably caused by spontaneous action potentials within the t-tubules (Purves and Sakmann 1974; Smith and Thesleff 1976). The morphological origin of irregularly discharging fibrillations is therefore the same as that of HRP uptake in the denervated diaphragm muscle. This suggests the interesting possibility that the processes triggering fibrillation potentials may be related to endocytic activity.

ACKNOWLEDGEMENTS

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Receptor-mediated Uptake of Horseradish Peroxidase in Innervated and Denervated Skeletal Muscle

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The *in vitro* uptake of [³H]inulin and horseradish peroxidase (HRP) has been studied in innervated and 6 days denervated extensor digitorum longus muscle of the mouse. Both markers were taken up at a higher rate in denervated muscle. The increase in uptake after denervation was, however, larger for HRP than for [³H]inulin. After 2 h incubation at 37°C, pH 7.3, in the presence of equimolar concentrations of HRP and [³H]inulin (approx. 2.1 μ M), the uptake of HRP was approx. 8 times as great as the uptake of [³H]inulin in the same innervated muscles. In denervated muscle the HRP uptake was approx. 19 times as great as the [³H]inulin uptake in the same muscles. Various possible explanations of these differences in uptake have been considered and tested experimentally. [³H]inulin uptake in skeletal muscle has previously been shown to obey bulk kinetics. The present investigation shows the HRP uptake to obey saturation kinetics. The HRP uptake shows dependency on divalent cations and is reduced if incubation is carried out at pH 6.4. The uptake of HRP, when used at a low, non-saturating concentration (10 μ g/ml approx. 0.25 μ M), is inhibited $\geq 60\%$ by yeast mannan (0.1 mg/ml), ribonuclease B (0.1 mg/ml, approx. 7.4 μ M), mannose (30 mM), monodansylcadaverine (1 mM), chloroquine (100 μ M), trifluoperazine (25 μ M) or maleic acid (2 mM). It is concluded that HRP is taken up in innervated and denervated skeletal muscle by a process of receptor-mediated endocytosis and that this uptake is under neurotrophic control. © 1985 Academic Press, Inc.

The endocytic uptake of [³H]inulin and horseradish peroxidase (HRP) in innervated and denervated skeletal muscle has been studied with radiochemical, biochemical, light- and electron-microscopical methods in a number of earlier publications from this laboratory [1–5]. The uptake of [³H]inulin has been shown to obey bulk kinetics [1]. The uptake of HRP, on the other hand, has not been characterized in detail, but previously published data [1] indicate that HRP is taken up at a much higher rate than [³H]inulin.

The aim of the present investigation was to study the difference in uptake between [³H]inulin and HRP in innervated and denervated skeletal muscle and to try to characterize the HRP uptake in more detail.

MATERIAL AND METHODS

All experiments were performed on innervated or 6-days denervated extensor digitorum longus (EDL) muscle of adult male NMRI mice, weighing about 30 g. Denervation was carried out by

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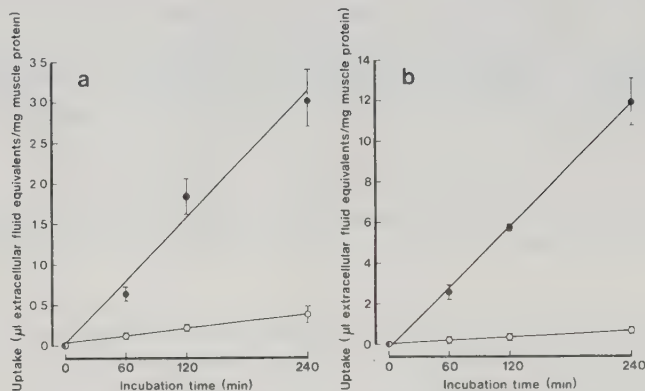


Fig. 1. The uptake of ●, horseradish peroxidase; ○, [^3H]inulin at equimolar concentrations (approx. $2.1 \mu\text{M}$) in the same (a) innervated; (b) denervated muscles. Incubation for 60–240 min at 37°C was followed by overnight washing at 4°C . Uptake is for both markers expressed as μl extracellular fluid equivalents taken up per mg muscle protein. Note difference in the ordinate scales of (a) and (b). Endogenous peroxidase activity has been subtracted. Mean values $\pm$ SE ($n=6$ for all points).

sectioning the deep peroneal nerve just above the knee, under diethyl-ether anesthesia. When innervated and denervated muscles were to be compared, denervation was performed unilaterally and the contralateral muscle was used as innervated control.

Animals were sacrificed by cervical dislocation and the EDL muscles were dissected and mounted on perspex holders (six muscles on each holder) kept in oxygenated Ringer's solution, pH 7.2–7.4 (composition in mM: NaCl, 135; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; Na_2HPO_4 , 1; NaHCO_3 , 15; glucose, 11; bubbled with 95% O_2 –5% CO_2) at room temperature. After mounting, the muscles were placed in 10 ml of an oxygenated incubation solution containing HRP and/or [^3H]inulin as extracellular markers. [^3H]inulin was always used at an activity of $2.5 \mu\text{Ci/ml}$ (approx. $2.1 \mu\text{M}$). A molecular weight of 40000 was assumed for HRP and $82.7 \mu\text{g/ml}$ was used as equimolar to the [^3H]inulin concentration. During incubation the solution was continuously bubbled with 95% O_2 –5% CO_2 supplied through a peristaltic pump to give standardized oxygenation and stirring conditions.

Incubations were normally carried out at 37°C . Incubations at 4°C were carried out using an ice–water mixture for cooling. Incubation times varied from 15 min to 4 h. When different incubation conditions were used or various substances were added to the incubation solution muscles were preincubated for approx. 40 min at 37°C in the absence of extracellular markers. Incubation at pH 6.4 was carried out in Ringer's solution adjusted to pH 6.4 by addition of HCl. In this experiment and in the experiment with calcium- and magnesium-free Ringer's solution the osmolarity was compensated by adjusting the NaCl concentration. In the other experiments no adjustments were made to compensate the osmolarity.

After incubation, the muscles were washed overnight in four changes (100–150 ml each) of oxygenated Ringer's solution at 4°C (in a cold-room). The muscles were then blotted dry, weighed, rapidly frozen on a glass dish on crushed dry ice and stored individually in sealed plastic tubes at -20°C . Muscles incubated at 4°C were used for determining the extracellular space. These muscles were not washed after the incubation but were briefly blotted on filter paper and then frozen immediately without weighing.

The muscles were later homogenized in 2 ml ice-cold 0.3 M sucrose using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) to yield a homogenate of approx. 0.5% muscle tissue (wet weight).

One ml of the homogenate was added to 10 ml Insta-Gel for determination of radioactivity by liquid scintillation counting. Counting efficiency was determined from an external standard with [^3H]toluene, using CCl_4 as quenching agent. The radioactivity in the incubation solutions was also deter-

mined and this data was used for converting muscle radioactivity content into μl extracellular fluid equivalents.

The muscles were assayed for peroxidase activity according to the method of Lundquist & Josefsson [6], using a 0.1 M maleic acid–NaOH buffer at pH 6.0 and a glucose oxidase concentration of 1 $\mu\text{g/ml}$ in the reagent solution. The incubation solutions were also assayed for peroxidase activity and this data was used for converting muscle HRP content into μl extracellular fluid equivalents.

Protein was measured according to the method of Lowry et al. [7] with bovine serum albumin as standard.

Chemicals

Special chemicals used were obtained from the following sources: [^3H]inulin (Batch 46 sp. act. 1.21 Ci/mmol) from the Radiochemical Centre, Amersham, Bucks, UK; Insta-Gel and [^3H]toluene from Packard Instrument Company, Inc., Downers Grove, Ill., USA; glucose oxidase (grade I) from Boehringer, Mannheim; chloroquine-diphosphate from Serva Feinbiochemica, Heidelberg; D(+)-mannose from British Drug Houses Ltd, Poole, Dorset, horseradish peroxidase (types II, VIII and IX), mannan (from baker's yeast), D-mannose-6-phosphate (grade I, disodium salt), monodansylcadaverine, ribonuclease A and B (from bovine pancreas, type XII-A and XII-B) and trifluoperazine-dihydrochloride from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Muscle Weight and Protein Content

The average wet weight of 70 innervated muscles used in the present study was 11.0 ± 0.1 mg (mean $\pm$ SE, $n=70$) and their protein content was 119.2 ± 3.1 mg protein/g muscle wet weight (mean $\pm$ SE, $n=70$). Contralateral, 6-days denervated muscles from the same animals had an average weight of 10.0 ± 0.1 mg (mean $\pm$ SE, $n=70$) and a protein content of 107.2 ± 3.4 mg protein/g muscle wet weight (mean $\pm$ SE, $n=70$).

Endogenous Peroxidase Activity

Endogenous peroxidase activity in the muscles represented the equivalents of between 2.0 and 4.9 ng HRP/mg muscle protein, depending on the specific activity of the HRP used. The endogenous peroxidase activity was 30–40% higher in denervated than in innervated muscles. The values presented for HRP uptake have been corrected for this endogenous peroxidase activity.

The Uptake of Horseradish Peroxidase and [^3H]inulin at 37°C

The uptake of HRP and [^3H]inulin at equimolar concentrations (approx. 2.1 μM) in the same muscles at 37°C is shown in fig. 1*a, b* for innervated and denervated muscle respectively. In order to illustrate the difference in uptake of the two markers, uptake is expressed as μl extracellular fluid equivalents taken up per mg muscle protein. Note difference in the ordinate scales of fig. 1*a, b*. The linear rates of uptake between 1 and 4 h for HRP and [^3H]inulin are 0.774 $\mu\text{l/mg}$ muscle protein/h and 0.086 $\mu\text{l/mg}$ muscle protein/h respectively in innervated muscle and 3.087 $\mu\text{l/mg}$ muscle protein/h and 0.152 $\mu\text{l/mg}$ muscle protein/h in

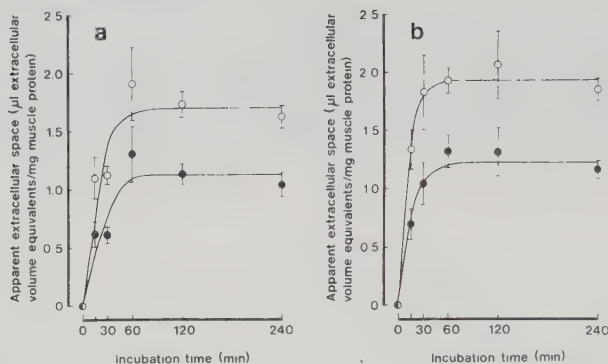


Fig. 2. Labelling of the apparent extracellular space at 4°C with ●, horseradish peroxidase; ○, [³H]inulin at equimolar concentrations (approx. 2.1 µM) in the same (a) innervated; (b) denervated muscles. The apparent extracellular space is for both markers expressed as µl extracellular volume equivalents labelled per mg muscle protein. Endogenous peroxidase activity has been subtracted. Mean values ± SE (*n*=6 for all points).

denervated muscle. The increase in uptake after denervation is thus larger for HRP than for [³H]inulin. This is also clear from the ratio of HRP uptake to [³H]inulin uptake in the same innervated or denervated muscles. After 2 h incubation this ratio is 8.3 ± 0.3 (mean ± SE, *n*=6) in innervated muscle and 19.0 ± 0.7 (mean ± SE, *n*=6) in denervated muscle.

Labelling of the Apparent Extracellular Space with Horseradish Peroxidase and [³H]Inulin at 4°C

The labelling of the apparent extracellular space with HRP and [³H]inulin at equimolar concentrations (approx. 2.1 µM) in the same muscles is shown in fig. 2a, b for innervated and denervated muscle respectively. The apparent extracellular space equilibrates at a similar rate with the two markers. The space labelled with [³H]inulin (approx. 1.750 µl/mg muscle protein in innervated muscle and 1.945 µl/mg muscle protein in denervated muscle), however, appears to be somewhat larger than that for HRP (approx. 1.160 µl/mg muscle protein in innervated muscle and 1.265 µl/mg muscle protein in denervated muscle).

Two hours incubation at 4°C gave a [³H]inulin content of $10\,003 \pm 636$ dpm/mg muscle protein (mean ± SE, *n*=6) in innervated muscle and $11\,908 \pm 1\,684$ dpm/mg muscle protein (mean ± SE, *n*=6) in denervated muscle. Corresponding values for HRP content were 77.6 ± 5.9 ng/mg muscle protein (mean ± SE, *n*=6) in innervated muscle and 89.9 ± 14.2 ng/mg muscle protein (mean ± SE, *n*=6) in denervated muscle. Washing the muscles for 4×1 h or overnight with four changes of Ringer's solution at 4°C reduced [³H]inulin and HRP content more than 95% in all cases.

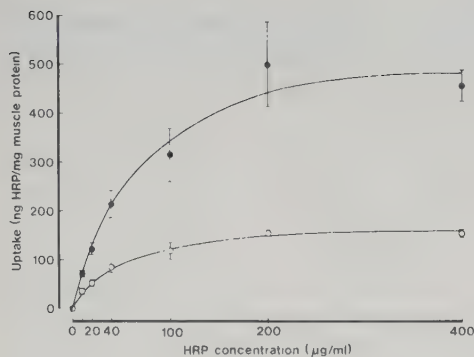


Fig. 3. The uptake of horseradish peroxidase in $\circ$, innervated; $\bullet$, denervated muscle during 2.5 h incubation at 37°C in the presence of different horseradish peroxidase concentrations. Uptake is expressed as ng horseradish peroxidase taken up per mg muscle protein. Endogenous peroxidase activity has been subtracted. Mean values $\pm$ SE ($n=4$ for all points).

Disappearance of Intracellular Horseradish Peroxidase and [^3H]Inulin at 37°C

Muscles were allowed to take up HRP and [^3H]inulin at equimolar concentrations (approx. 2.1 μM) for 2.5 h at 37°C. After overnight washing at 4°C in four changes of Ringer's solution, incubation was continued for an additional 2 h at 37 or 4°C (control muscles) in Ringer's solution containing no marker. The loss of HRP during this incubation period at 37°C amounted to $25.0 \pm 10.1\%$ (mean $\pm$ SE, $n=6$) from innervated muscle and $24.7 \pm 11.8\%$ (mean $\pm$ SE, $n=6$) from denervated muscle. Corresponding values for [^3H]inulin in the same muscles were $36.2 \pm 4.3\%$ (mean $\pm$ SE, $n=6$) from innervated muscle and 36.6 ± 9.4 (mean SE, $n=6$) from denervated muscle.

Measurements of peroxidase activity in the incubation solutions from the second incubation at 37°C (results not shown) showed that some, but not all, peroxidase activity lost from the muscles could be recovered in the incubation solution. This indicates that the loss of activity from the muscles is due partly to secretion and partly to degradation of the enzyme.

Saturation of Horseradish Peroxidase Uptake

The uptake of HRP during 2.5 h at 37°C in the presence of different HRP concentrations is shown in fig. 3 for innervated and denervated muscle. As can be seen in fig. 3, the HRP uptake in both innervated and denervated muscle becomes saturated at HRP concentrations above 100–200 $\mu\text{g/ml}$ (approx. 2.5–5 μM). The uptake in denervated muscle is considerably larger than that in innervated muscle at all concentrations of marker.

In the experiments reported below, a low, non-saturating HRP concentration (10 $\mu\text{g/ml}$ approx. 0.25 μM) was chosen and incubation was carried out for 2.5 h at 37°C. Under these conditions the HRP uptake in 32 innervated control muscles was 44.8 ± 1.8 ng/mg muscle protein (mean $\pm$ SE, $n=32$). In the same muscles the [^3H]inulin uptake amounted to $1\,100.4 \pm 34.5$ dpm/mg muscle protein (mean $\pm$ SE,

Table 1. *The uptake of different horseradish peroxidase isoenzymes in innervated skeletal muscle*

HRP type	HRP uptake (ng/mg muscle protein)
	Mean $\pm$ SE (n)
Type II (mixture of isoenzymes)	44.8 $\pm$ 1.8 (32)
Type VIII (acidic isoenzyme)	45.0 $\pm$ 4.0 (6)
Type IX (basic isoenzyme)	52.9 $\pm$ 8.9 (6)

The different isoenzymes were all used at a concentration of 10 μ g/ml. Uptake is expressed as ng horseradish peroxidase taken up per mg muscle protein during a 2.5 h incubation at 37°C.

$n=32$), or 0.208 ± 0.007 μ l extracellular fluid/mg muscle protein. If the [3 H]inulin uptake is taken to represent bulk uptake, then the bulk component in the HRP uptake would account for 2.1 ng/mg muscle protein, i.e., less than 5% of the total HRP uptake.

Uptake of Different Horseradish Peroxidase Isoenzymes

The uptake of different isoenzymes of HRP in innervated muscle is shown in table 1. The acidic and basic isoenzymes are expected to carry different overall charges at neutral pH but are nevertheless taken up at similar rates.

Incubation Conditions and Substances that Influence the Uptake of Horseradish Peroxidase

A number of different incubation conditions and substances are able to interfere with HRP uptake, as is shown in tables 2 and 3 for innervated and denervated muscle. Uptake is expressed as % of the uptake in paired control muscles from the same animals incubated in normal Ringer's solution.

DISCUSSION

Both [3 H]inulin and HRP have been extensively used as extracellular markers for studying endocytosis in a variety of cells. Both have often been assumed to be taken up primarily by fluid-phase pinocytosis. However, for HRP a receptor-mediated uptake has been demonstrated in rat and rabbit alveolar macrophages [8, 9] and HRP has also been found to bind to mannose-specific receptors on various cell types in fixed, frozen sections from different rat tissues [10–12]. The rapid clearance of HRP following infusion into the rat has also been reported to be mediated by specific recognition sites [13].

The present study describes a large quantitative difference between the uptake of HRP and [3 H]inulin in innervated and denervated skeletal muscle. The uptake of both these markers is believed to occur into skeletal muscle fibres rather than

Table 2. The influence of different incubation conditions and substances on the uptake of horseradish peroxidase in innervated muscle

Incubation condition or substance added	HRP uptake (% of control)
	Mean $\pm$ SE, $n=6$
pH 6.4	18.9 $\pm$ 2.4
0 Ca ²⁺ , 0 Mg ²⁺	37.2 $\pm$ 1.2
Yeast mannan 0.1 mg/ml	19.6 $\pm$ 2.7
Yeast mannan 1.0 mg/ml	7.1 $\pm$ 0.6
HRP type VIII + yeast mannan 0.1 mg/ml	13.6 $\pm$ 0.9
HRP type IX + yeast mannan 0.1 mg/ml	18.5 $\pm$ 2.5
Ribonuclease A 0.1 mg/ml approx. 7.4 μ M	97.1 $\pm$ 10.2
Ribonuclease B 0.1 mg/ml approx. 7.4 μ M	40.6 $\pm$ 3.5
Mannose 1 mM	86.9 $\pm$ 5.6
10 mM	48.7 $\pm$ 6.0
30 mM	21.7 $\pm$ 0.7
50 mM	14.9 $\pm$ 0.9
Mannose-6-phosphate 30 mM	52.1 $\pm$ 2.4
Sucrose 10 mM	74.9 $\pm$ 6.0
30 mM	68.0 $\pm$ 8.8
Chloroquine 100 μ M	4.4 $\pm$ 0.7
1 mM	0.9 $\pm$ 0.5
Monodansylcadaverine 100 μ M	80.2 $\pm$ 5.5
1 mM	0.0 $\pm$ 0.4
Trifluoperazine 25 μ M	39.6 $\pm$ 2.9
Maleic acid 2 mM	21.7 $\pm$ 3.7

Horseradish peroxidase was used at a concentration of 10 μ g/ml (approx. 0.25 μ M). Uptake is expressed as % of the uptake in paired control muscles from the same animals incubated in normal Ringer's solution.

into other cellular elements of the muscle. In denervated muscle the *in vivo* uptake of HRP has been demonstrated to occur segmentally in muscle fibres by both histochemical and ultrastructural methods [2, 4, 5]. The uptake in innervated muscle has not been demonstrated with morphological methods. However, the size of uptake in innervated muscle makes it unlikely that it could be accounted for by endocytic activity of other cellular elements in muscle than the muscle fibres themselves. Various mononuclear phagocytes and fibroblasts have been reported to take up fluid-phase markers at rates of 0.020–0.096 μ l/10⁶ cells/h (0.250–1.200 μ l/mg protein/h) and 0.027–0.086 μ l/10⁶ cells/h (0.059–0.190 μ l/mg protein/h) respectively [14, 15]. In the present study the [³H]inulin uptake was 0.086 μ l/mg muscle protein/h in innervated muscle. This uptake has previously been shown to obey bulk kinetics [1]. If this uptake was due only to mononuclear phagocytes then 7–34% of the total muscle protein would have to be derived from these cell types. If the uptake was due only to fibroblasts then >45% of the total muscle protein would have to be derived from fibroblasts. This rough calculation suggests that it is unlikely that the uptake in innervated muscle is due only to the

Table 3. *The influence of different substances on the uptake of horseradish peroxidase in denervated muscle*

Substance added	HRP uptake (% of control)
	Mean $\pm$ SE, $n=6$
Yeast mannan 1 mg/ml	4.6 $\pm$ 1.5
Chloroquine 1 mM	0.0 $\pm$ 0.7
Monodansylcadaverine 1 mM	0.0 $\pm$ 0.7

Horseradish peroxidase was used at a concentration of 10 μ g/ml (approx. 0.25 μ M). Uptake is expressed as % of the uptake in paired control muscles from the same animals incubated in normal Ringer's solution.

endocytic activity of mononuclear phagocytes and fibroblasts present in the muscle. From the values of the [3 H]inulin extracellular space (1.750 μ l/mg muscle protein) and the [3 H]inulin uptake (0.086 μ l/mg muscle protein/h) in innervated muscle it may be calculated that this endocytic process turns over a volume equivalent to the extracellular space of the muscle in little more than 20 h. In denervated muscle the corresponding time is less than 13 h.

The main aim of the present investigation was to try to explain the large difference in uptake between [3 H]inulin and HRP in skeletal muscle. Several possible explanations have been considered. For example, HRP could be unspecifically adsorbed to the cell membrane and thereby be taken up at a higher rate than [3 H]inulin. This possibility, however, appears unlikely for two reasons. Firstly, if a significant amount of HRP was adsorbed to cell membranes then the apparent extracellular space labelled with this marker would be expected to be disproportionately large. In the present study the opposite was observed, i.e., the apparent extracellular space was found to be smaller for HRP than for [3 H]inulin. Secondly, acidic and basic isoenzymes of HRP are taken up at a similar high rate, despite the fact that at neutral pH the two isoenzymes would be expected to carry different overall charges and therefore show different affinities for charges on cell membranes.

A second possibility considered was that the difference in net uptake of HRP and [3 H]inulin could be due to the two markers having different elimination rates. This possibility is rejected, since the results indicate that both markers disappear from the muscles with similar rates.

The most likely explanation of the large difference in the uptake of HRP and [3 H]inulin is that HRP is taken up by a receptor-mediated endocytic process. This conclusion is based on the saturation kinetics of the HRP uptake (fig. 3) and on the ability of different incubation conditions and substances to inhibit the HRP uptake (tables 2, 3). The number of receptors is assumed to be relatively small, since binding does not influence the apparent size of the extracellular space. To

explain the high uptake rate it is assumed that the receptors are internalized and re-utilized at a high rate.

HRP is a glycoprotein rich in mannose residues [16, 17], and the inhibitory effects of yeast mannan and mannose on the HRP uptake suggests that these mannose residues are important for the recognition process. This is also suggested by the fact that other glycoproteins rich in mannose residues, such as ribonuclease B, can inhibit the HRP uptake. The non-glycoprotein enzyme ribonuclease A showed no such inhibitory effect. Since yeast mannan (mannose polymer) and glycoproteins rich in mannose (ribonuclease B) are much more potent inhibitors than is mannose (table 3), this may suggest that binding of HRP involves recognition of sequences of mannose, possibly in combination with other sugar residues. This is in agreement with a recent study on isolated rat hepatic reticuloendothelial cells [18] which suggested a minimum required oligosaccharide structure for binding and endocytosis of glycopeptides and glycoproteins.

Removal of divalent cations or lowering the pH of the incubation solution greatly reduces the uptake of HRP. This suggests that the HRP binding site belongs to the class II receptors in the classification suggested by Kaplan [19] and Anderson & Kaplan [20]. The uptake of HRP is also inhibited in the presence of monodansylcadaverine, chloroquine or trifluoperazine. Monodansylcadaverine has been suggested to inhibit receptor-mediated endocytosis by inhibition of the enzyme transglutaminase which may be involved in cross-linking proteins during clustering of ligand-receptor complexes in coated pits ([21], but see also [20, 22, 23] for discussion). Chloroquine and other weak bases have been suggested to inhibit receptor-mediated endocytosis by increasing the pH of lysosomes or other cellular compartments, where uncoupling of ligand from receptor may occur, thereby inhibiting intracellular delivery of ligand [see 24]. Trifluoperazine, an inhibitor of the calcium-regulatory protein calmodulin, also appears to be able to inhibit receptor-mediated internalization of ligands along the coated vesicle pathway [see 22].

The inhibitory effect of maleic acid on HRP uptake in skeletal muscle was discovered by coincidence. The mechanism behind this inhibitory effect remains to be elucidated.

The uptake of the [^3H]inulin and HRP in skeletal muscle appears to be under neurotrophic control. Denervation results in an increase in the uptake of both markers. Interestingly, however, the increase in HRP uptake is larger than the increase in [^3H]inulin uptake. After denervation HRP uptake has been localized in segments of muscle fibres which also contain high activities of lysosomal enzyme [2, 4]. Recently, these segments have been found to be localized almost exclusively to the endplate region of the muscle fibres [5].

It appears then that there exists on skeletal muscle fibres receptors for glycoproteins rich in mannose and a mechanism for internalization of these glycoproteins. Denervation speeds up this process and the uptake becomes concentrated primarily to the endplate region of the muscle fibres. This creates segments with

high uptake activity and high lysosomal enzyme content. Such segments have been seen to occur in the denervated mouse hemidiaphragm up to 7 weeks after denervation (unpublished observations). Similarly, the increased uptake of HRP in denervated mouse EDL muscle has been followed up to 7 weeks after denervation (unpublished observations).

The question about the function of the described phenomena cannot receive any definite answer at this time point. Many extracellular glycoproteins as well as many membrane glycoproteins are rich in mannose residues and could have affinity for the described uptake system. Many lysosomal enzymes are also rich in mannose content and mannose-recognizing receptors could conceivably be involved in the uptake and/or intracellular transport of such enzymes. A mannose-6-phosphate receptor, which may also be involved in uptake/transport of certain lysosomal enzymes, has also recently been shown to occur on skeletal muscle cells [25]. It has also been suggested recently that lysosomal enzymes in denervated skeletal muscle derive from degenerating axons at the myoneural junction [26]. Such degenerative processes near the muscle fibres have also been suggested to trigger many of the denervation changes seen in skeletal muscle [27, 28].

A different possibility is that the endplate region of denervated muscle becomes involved in secretory activities. If HRP has affinity to some membrane components involved in this process it may become internalized during recycling of these membrane components.

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AN ULTRASTRUCTURAL STUDY OF THE SEGMENTAL UPTAKE
OF HORSERADISH PEROXIDASE IN THE ENDPLATE REGION
OF DENERVATED SKELETAL MUSCLE FIBRES

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SUMMARY

The segmental uptake of horseradish peroxidase (HRP) in the endplate region of denervated skeletal muscle fibres has been studied ultrastructurally using a method for selecting single muscle fibres with high segmental peroxidase staining from denervated mouse tibialis anterior muscle.

Segments containing large peroxidase positive phagosomes could already be seen 10-15 min after i.v. injection of HRP. Such segments were still present 24 hours after HRP injection. The localization of phagosomes, deep in the fibres rather than immediately under the sarcolemma, suggests that the uptake occurs from t-tubuli. Vivid proliferation of t-tubuli, consisting of vesiculation, enlargement and encircling of cytoplasmic components, was also observed. The HRP accumulates in phagosomes of varying size and shape. Similar membrane-limited bodies without or with very weak peroxidase staining were also observed. The peroxidase positive phagosomes participate in autophagic processes as suggested by their content of undegraded cellular material. Golgi profiles, which occurred deep in the muscle fibres, and enlarged components of the sarcoplasmic reticulum were frequently encountered in the segments. Myofibrillar degeneration occurs in the segments and progresses with time after denervation.

The described segments may be related to the increased membrane turnover in denervated muscle fibres and/or they may be related to processes aimed at establishing new synaptic contacts.

Key words: Degeneration - Denervation - Endocytosis - Endplate - Horseradish peroxidase - Skeletal muscle - Ultrastructure

INTRODUCTION

In two previous publications (Libelius 1974; 1975) evidence was presented for endocytic uptake of a cobra neurotoxin in mouse skeletal muscle. Since then endocytosis has been studied with a number of different markers and techniques in normal innervated muscles, dystrophic muscles, denervated muscles (Libelius et al. 1978 a; 1978 b; 1979 a; 1979 b; 1981; Sellin et al. 1980; Libelius and Tågerud 1984; Tågerud and Libelius 1984; 1985) and in muscles paralyzed by neural application of batrachotoxin or tetrodotoxin (Boegman and Scarth 1981). Recently the uptake of horseradish peroxidase (HRP) in skeletal muscle has been shown to occur by a process of receptor-mediated endocytosis (Tågerud and Libelius 1985). Denervation results in an increased uptake of HRP and the increase in uptake occurs in segments localized near the endplate region of the muscle fibres (Libelius and Tågerud 1984). The HRP positive segments have also been shown histochemically to stain for the lysosomal enzyme acid phosphatase (Tågerud and Libelius 1984) and biochemically the endplate region of the denervated hemidiaphragm exhibits higher lysosomal enzyme activities than the surrounding muscle (Libelius and Tågerud 1984).

An ultrastructural study of the HRP uptake in denervated muscle has been performed previously (Libelius et al. 1979b). This study was, however, carried out on blocks of muscle tissue which sometimes resulted in uneven staining for peroxidase in the centre of the blocks. The staining for HRP was thus variable and the yield of material for ultrastructural study was rather limited.

The present investigation was undertaken in order to study the ultrastructural appearance of the segmental uptake of HRP in denervated muscle in a larger amount of material. In order to gain more information about how the uptake occurs, how segments are formed, and which relationship they have to myofibrillar degeneration the process was studied at different times after denervation and at different times after labelling with HRP. In a subsequent publication (Tågerud et al. submitted) the effects of the lysosomotropic amine chloroquine on the appearance of these segments will also be described.

In order to obtain a good yield of homogeneously stained and embedded material for an ultrastructural study, a method was developed for selecting single fibres with high segmental peroxidase staining from the denervated tibialis anterior muscle of the mouse.

MATERIALS AND METHODS

All experiments were performed on the tibialis anterior muscle of adult male NMRI mice, weighing about 30 g. Denervation was carried out under diethyl-ether anaesthesia by sectioning the deep peroneal nerve just above the knee. The animals were sacrificed 4 or 12-14 days after denervation. At various times (10-15 min, 2 hours, 10 hours or 24 hours) before sacrifice 20 mg of HRP dissolved in 0.2 ml of 0.9% saline was injected into a tail vein.

Fixation of muscles

The animal was anaesthetized by an intraperitoneal injection of pentobarbital. After pinning the animal in an extended position, the thoracic cavity was opened and a cannula was inserted into the aorta through the heart. The cannula was fixed in position with a ligature and after cutting open the right auricle the animal was perfused with about 20 ml of 0.9% saline at room temperature followed by about 30 ml of fixative at room temperature. The composition of the fixative was: glutaraldehyde, 2%; paraformaldehyde, 1%; $\text{CaCl}_2 \cdot (2\text{H}_2\text{O})$, 20 mg/l; phosphate buffer, 0.12 M, pH 7.3). The time after HRP injection was counted as the time that elapsed from the HRP injection until perfusion with fixative was commenced. After the perfusion the skin was removed from the lower part of the hind limb and the tibialis anterior muscle was fixed in situ by injecting a small volume of cold fixative into the muscle, using a 0.4 mm hypodermic needle. After this the muscle was dissected, pinned onto Sylgaard resin and fixed by immersion for an additional 2 hours at 4°C . Following fixation the muscles were rinsed for 1-2 days at 4°C in a wash solution of the following composition: sucrose, 7.5%; $\text{CaCl}_2 \cdot (2\text{H}_2\text{O})$, 20 mg/l; phosphate buffer, 0.12M, pH 7.3.

Preparation of single muscle fibres containing segments with high peroxidase activity

Using a dissecting microscope and fine forceps single muscle fibres or small bundles of fibres were teased off from the muscles. These were placed in small drops of a staining solution for peroxidase on a microscope slide. The composition of the staining solution was: 3,3'-diaminobenzidine hydrochloride (1 mg/ml), glucose oxidase (0.2 mg/ml) and D-glucose (2 mg/ml), all dissolved in the wash solution described above. After several minutes of staining at room temperature the fibres were inspected for peroxidase staining under a dissecting microscope and under a light microscope. Fibres containing segments with dense peroxidase staining of granular appearance

were, when necessary, further separated from other fibres and then collected in the wash solution.

The selected fibres were rinsed twice in a 0.2 M phosphate buffer, pH 7.3, postfixed in 2% OsO_4 in a 0.1 M phosphate buffer, pH 7.3, for 1 hour at room temperature, rinsed in 2.4% NaCl, dehydrated and embedded in Polarbed 812. All handling of the single fibres was carried out under a dissecting microscope.

Semithin sections (1-2 μm) were inspected under a light microscope. Ultrathin sections from selected areas were stained with lead citrate and uranyl acetate and examined in a Jeol CX100 electron microscope.

Chemicals

Special chemicals used were obtained from the following sources: horseradish peroxidase (type II) from Sigma Chemical Co., St. Louis, MO, U.S.A.; 3,3'-diaminobenzidine tetrahydrochloride from British Drug Houses Ltd., Poole, U.K.; glucose oxidase (grade I) from Boehringer, Mannheim, F.R.G.; glutaraldehyde (8% aqueous, E.M. grade) from Polysciences Inc., Warrington, PA., U.S.A.

RESULTS

General

Muscle fibres containing segments with high peroxidase activity were collected from muscles taken at various times after denervation and HRP injection as shown in Table I.

TABLE I: Number of fibres containing segments with high peroxidase activity studied at various times after denervation and HRP injection.

Days after denervation	Time after HRP injection	Number of segments studied
4	2 hours	16
14	10-15 min.	4
12-14	2 hours	19
14	10 hours	13
14	24 hours	16
		Total: 68

The appearance, in the light microscope, of an intact muscle fibre containing a segment with high peroxidase activity is shown in Fig. 1. A semithin section of a similar segment is shown in Fig. 2.

The ultrastructural results presented were evaluated from about 950 electronmicrographs taken from the 68 segments included in this study. Each segment was photographed at low magnification for overview and at higher magnifications in selected regions. The appearance of two segments at low magnification in the electron microscope is shown in Figs. 3 and 4.

Ultrastructure.

Relationship of segments to the endplate region

In the 68 segments studied, post-synaptic folds, or remnants thereof, were visible in the vicinity of 12 segments (17.6%). Part of a segment with HRP uptake near post-synaptic folds is shown in Fig. 5.

Myofibrillar degeneration in segments.

The occurrence of myofibrillar degeneration in the segments after denervation was rather variable and was not observed in all segments. However, it occurred with typical localization and frequently enough to be considered a feature of the segments. Signs of myofibrillar degeneration were observed in 2 out of 16 segments (12.5%) at 4 days after denervation and in 35 out of 52 segments (67.3%) at 12-14 days after denervation. The difference between these proportions is highly significant ($p < 0.001$; χ^2 -test).

Degeneration was usually seen in the centre of a HRP segment but was sometimes restricted to the outer boundary of the segment. Signs of degeneration include loss of cross striation. Examples of segments with central or more focal myofibrillar degeneration can be seen in Figs. 3, 5, 6 and 8.

T-tubular system

A vivid proliferation of the t-tubular system was observed in the segments (Figs. 7-10 and 21). Elaborate networks of vesicular profiles and longitudinally oriented t-tubuli were frequently seen. T-tubular profiles with an enlarged appearance were also common (Figs. 7 and 8). T-tubular profiles also appeared to encircle cytoplasmic areas and/or cytoplasmic bodies (Figs. 9 and 21).

The t-tubuli were well labelled with peroxidase in fibres collected from muscles taken 10-15 min after HRP injection (Figs. 11 and 12). At 10-24 hours after HRP injection the labelling of the t-system was uneven. Some areas showed no labelling (Figs. 13 and 15) whereas other areas showed some labelling of t-tubuli (Figs. 14 and 20).

Sarcoplasmic reticulum

In the vicinity of segments enlarged components of the sarcoplasmic reticulum could frequently be observed with a characteristic fine granular appearance. These were encountered in the location of terminal cisternae (Fig. 11), under the sarcolemma (not shown) and in the vicinity of areas with t-tubular proliferation and peroxidase-positive phagosomes (Figs. 12 and 13). These fine granular bodies commonly had a rather irregular shape.

Where the t-system was oriented longitudinally sarcoplasmic reticulum components could occasionally be seen to be apposed to a t-tubule.

Golgi apparatus

Golgi profiles were noted in the sarcoplasm near the nuclear poles as in normal muscle. Golgi profiles were also frequently encountered deeper

in the fibres in association with the segments of high peroxidase content (Figs. 14 and 15). The Golgi cisternae were never seen to contain the peroxidase label.

Peroxidase-containing phagosomes

Peroxidase positive phagosomes occurred in the segments with a large variation in size and shape from small spherical to very large and irregular (Figs. 3, 4, 6, 9-10 and 18). Large peroxidase positive phagosomes could already be seen in segments collected from muscles taken 10-15 min after HRP injection (Fig. 18). Particularly in segments of fibres taken 10-24 hrs after HRP injection, many phagosomes were seen to contain undegraded material (Figs. 13-14, 16-17 and 20). Occasionally structures resembling mitochondria could be identified inside such phagosomes (Fig. 16). Phagosomes in the process of engulfing cytoplasmic components can be seen in Figs. 9, 13, 16 and 17.

Proliferating t-tubuli could frequently be seen close to peroxidase-positive phagosomes (Figs. 7-10). The phagosomes sometimes appeared to be in contact with t-tubuli and especially at 4 days after denervation, when most phagosomes were rather small, it was difficult to differentiate phagosomes from what appeared to be enlarged t-tubular profiles (Figs. 7-8).

Other membrane-limited bodies

Other membrane-limited bodies observed were of multivesicular appearance, usually containing peroxidase staining (Fig. 19), and bodies of similar shape and size to peroxidase containing phagosomes but with weaker staining or unstained (Figs. 3, 9, 12, 16, 18 and 20-22).

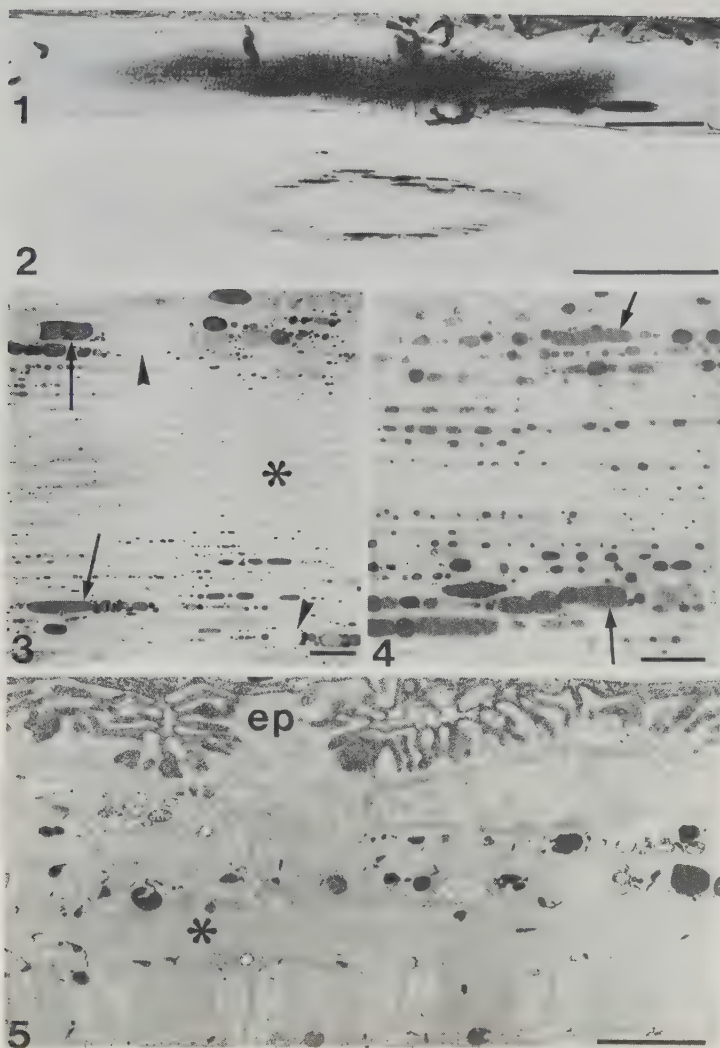


Fig. 1 Unsectioned muscle fibre containing a segment with high HRP uptake. 14-days denervated, 2 hrs after HRP injection. Bar = 50 μ m. Fig. 2 Semithin section of a fibre similar to the one shown in Fig. 1. 12-days denervated, 2 hrs after HRP injection. Bar = 50 μ m. Figs. 3-4 Two segments with high HRP uptake at low magnification in the electron microscope. Note large peroxidase positive phagosomes (some denoted by arrows), vacuoles showing weaker or no staining (arrowheads) and myofibrillar degeneration in the centre of the fibre in Fig. 3 (asterisk). 14-days denervated, 2 hrs (Fig. 3) and 24 hrs (Fig. 4) after HRP injection. Bars = 5 μ m. Fig. 5 Fibre showing segmental HRP uptake in the endplate region (ep). Note presence of localized myofibrillar degeneration (asterisk). 14-days denervated, 2 hrs after HRP injection. Bar = 2 μ m.

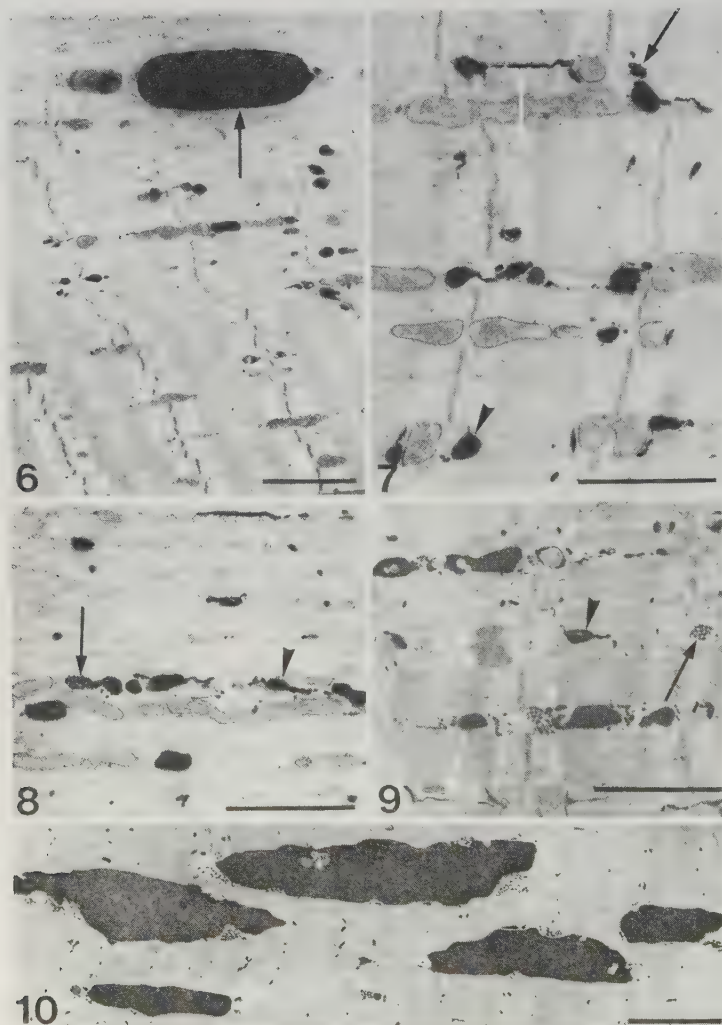
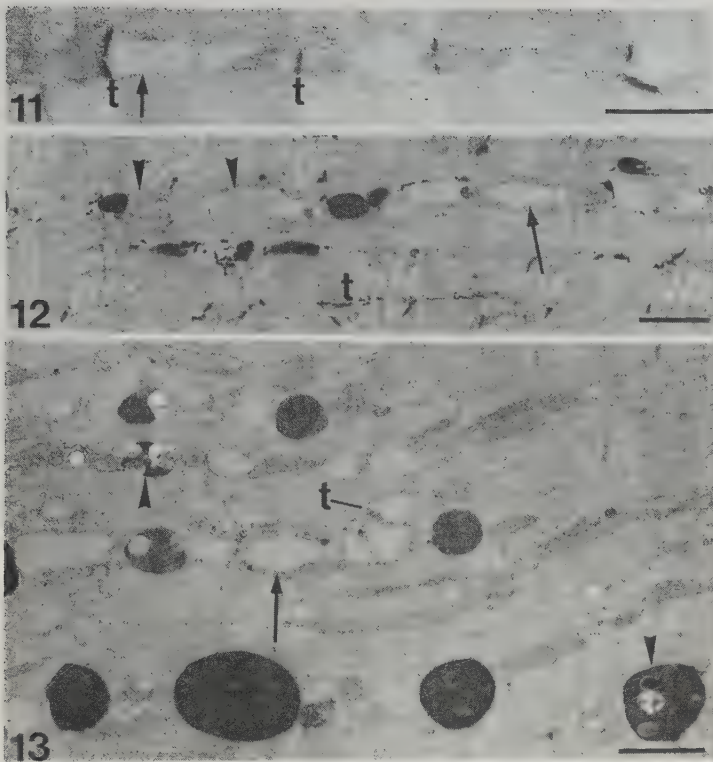
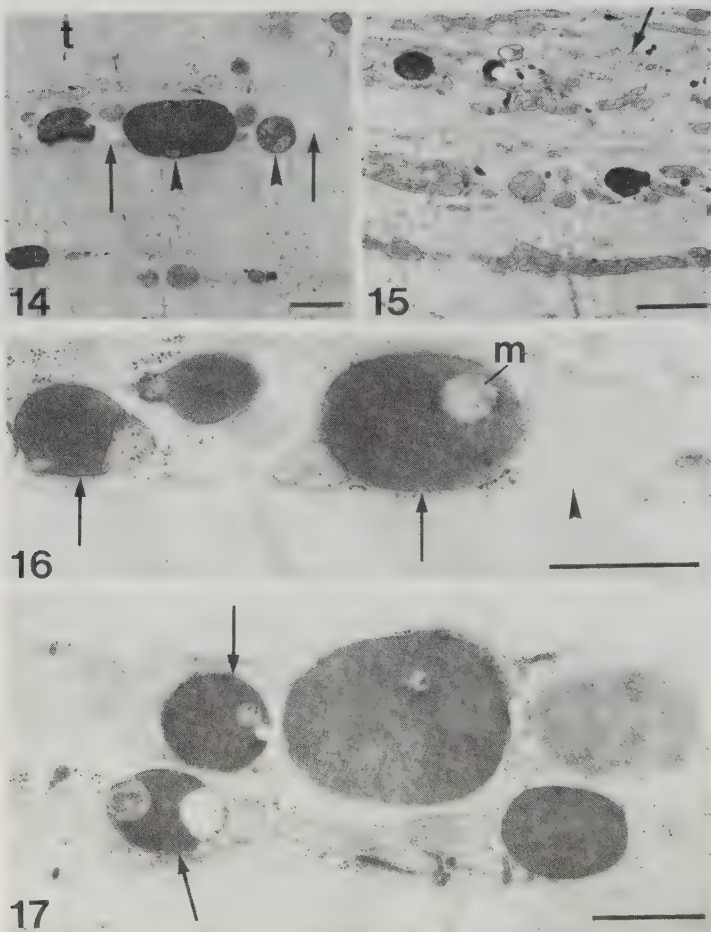


Fig. 6 Large peroxidase positive phagosome (arrow) located in an area of localized myofibrillar degeneration at the periphery of a muscle fibre. 14-days denervated, 24 hrs after HRP injection. Bar = 2 μ m. **Figs. 7-9** t-tubular proliferation seen as networks of vesicular profiles (black arrows), longitudinal orientation of t-tubuli (white arrows), enlarged t-tubuli or phagosomes in apparent contact with t-tubuli (black arrowheads) and t-tubuli encircling cytoplasmic components (white arrowhead in Fig. 9). Note also the myofibrillar degeneration in Fig. 8. 4-days (Figs. 7-8) and 14-days (Fig. 9) denervated, 2 hrs after HRP injection. Bars = 2 μ m. **Fig. 10** Very large and irregular phagosomes with nearby prominent vesicular profiles. 14-days denervated, 2 hrs after HRP injection. Bar = 2 μ m.



Figs. 11-13 Enlarged sarcoplasmic reticulum components (some denoted by arrows) in the location of terminal cisternae (Fig. 11) and in the vicinity of areas with t-tubular proliferation and peroxidase positive phagosomes (Figs. 12-13). Note phagosomes containing undegraded material or in the process of engulfing cytoplasmic components (arrowheads in Fig. 13) and other weakly stained or unstained membrane-limited bodies (arrowheads in Fig. 12). 14-days denervated, 10-15 min (Figs. 11-12) and 24 hrs (Fig. 13) after HRP injection. Note also well labelled t-tubuli (t) already 10-15 min after HRP injection (Figs. 11-12) and absence of t-tubular labelling 24 hrs after HRP injection (Fig. 13). Bars = 1 μ m.



Figs. 14-15 Golgi profiles (arrows) deep in muscle fibres near peroxidase positive phagosomes. Note also presence of undegraded material in phagosomes (arrowheads in Fig. 14). 14-days denervated, 24 hrs after HRP injection. Some t-tubuli (t) are still labelled with HRP (Fig. 14) although most t-tubuli contain no label. Bars = 1 μ m. Figs. 16-17 Phagosomes containing undegraded material or in the process of engulfing cytoplasmic components (arrows). Note structure resembling mitochondrion (m) inside phagosome in Fig. 16 and weakly stained or unstained membrane-limited body (arrowhead in Fig. 16). 14-days denervated, 10 hrs after HRP injection. Bars = 1 μ m.

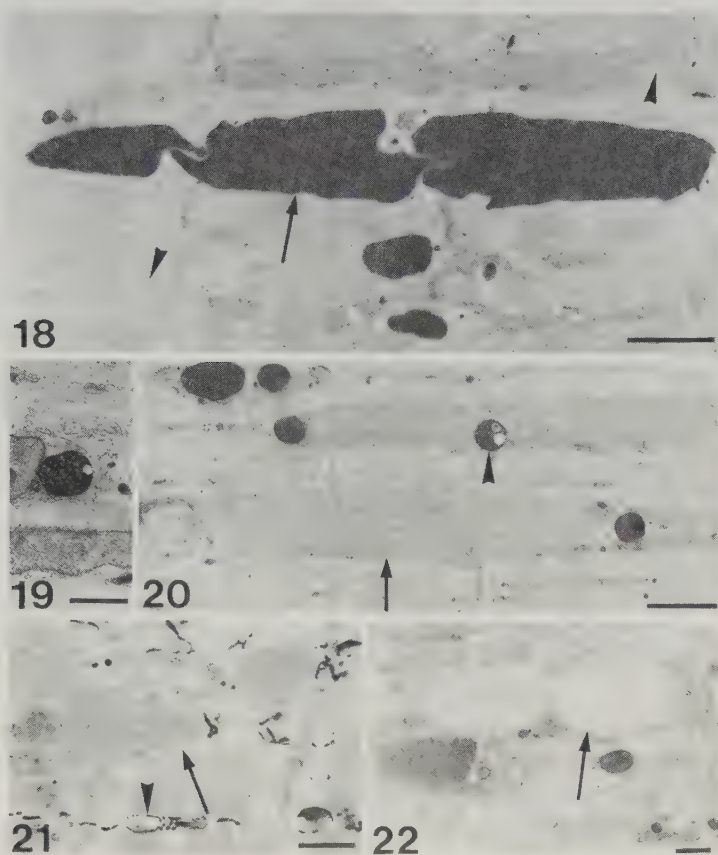


Fig. 18 Large irregular phagosomes (one denoted by arrow) filled with HRP already 10-15 min after injection. Note also membrane-limited bodies with weaker staining or unstained (arrowheads). 14-days denervated, 10-15 min after HRP injection. Bar = 1 μ m. Fig. 19 Peroxidase positive body of multivesicular appearance. 14-days denervated, 2 hrs after HRP injection. Bar = 0.5 μ m. Figs. 20-22 Membrane-limited bodies (some denoted by arrows) which are of similar size and shape to peroxidase positive phagosomes but with weaker staining or unstained. Note also presence of undegraded material in peroxidase positive phagosome (arrowhead in Fig. 20) and encircling of cytoplasmic area by t-tubuli (arrowhead in Fig. 21). 14-days denervated, 10 hrs (Fig. 20), 2 hrs (Fig. 21) and 24 hrs (Fig. 22) after HRP injection. Bars = 1 μ m.

DISCUSSION

Evaluation of the method for selecting single fibres with high segmental peroxidase staining

The method for selecting single fibres with high segmental peroxidase staining was developed because the segments are non-randomly distributed in denervated muscles (Libelius and Tågerud 1984; Tågerud and Libelius 1984) and because blocks of muscle tissue give uneven staining for peroxidase in the centre of the blocks (Libelius et al. 1979b). These difficulties are overcome by dissecting single fibres or small bundles of fibres from fixed muscle. The fibres are then stained for peroxidase and checked for segmental HRP uptake both under a dissecting and a light microscope. Only fibres with segments of high peroxidase staining of granular appearance are further processed, thus giving a good yield of material for ultrastructural study. Since staining and embedding are performed on single fibres or small bundles of fibres diffusion barriers are minimized giving homogeneously stained and embedded material.

The main disadvantage of the method is that there may be some risk of damaging the fibres mechanically during the preparation. To minimize this risk the muscles were carefully fixed by perfusion, injection of fixative into the muscle in situ and immersion in fixative for an additional two hours. Handling of the fibres was carried out under a dissecting microscope using fine-tipped forceps or thin hypodermic needles. Care was taken to apply the instruments only to the ends of the fibres avoiding touching the area of the segment.

Degeneration in the segments

In denervation atrophy several reports have demonstrated that myofibrillar disintegration occurs by a loss of myofilaments in the periphery of myofibrils (Pellegrino and Franzini 1963; Shafiq et al. 1967; Miledi and Slater 1969; Stonnington and Engel 1973; Tomanek and Lund 1973; Engel and Stonnington 1974). This atrophic process may also be going on in the segments with high HRP uptake but frequently pronounced disintegration with complete loss of cross striation is observed. This degenerative process progresses with time since signs of degeneration were significantly more common 14 days after denervation than 4 days after denervation. The cause of the pronounced degeneration in the segments is not clear but it may be related to the high activities of lysosomal enzymes which have previously been shown to occur in the segments (Libelius and Tågerud 1984; Tågerud and Libelius 1984). However, other possible causes of degeneration such

as an altered ionic milieu in the segments cannot be excluded. The active degenerative process appears to be specifically related to the segments and does not provide an obvious explanation of the general fibre atrophy that occurs after denervation.

The myofibrillar degeneration occurs primarily at the centre of the segments, producing lesions somewhat similar to those encountered in target fibres. Since target fibres are seen in denervated muscle (Engel 1961) and/or in muscles becoming reinnervated (Dubowitz 1967) targets may correspond to the segments described here with high HRP uptake.

Uptake of HRP and formation of phagosomes

Biochemical studies have recently shown that the HRP uptake in innervated and denervated skeletal muscle occurs by a receptor-mediated mechanism (Tågerud and Libelius 1985). A previous ultrastructural study suggested that the uptake of HRP in segments of denervated muscle fibres occurs by endocytosis (micropinocytosis) from t-tubuli (Libelius et al. 1979b). In the present study a vivid proliferation of the t-system was observed in the segments with high HRP uptake. HRP-labelled vesicular profiles appeared to derive from the t-system and these vesicles could frequently be seen close to larger peroxidase-positive phagosomes. Coated vesicles, which are often observed in receptor-mediated uptake processes, were not prominent in the segments although such vesicles have been observed in a previous study (Libelius et al. 1979b) and in studies of HRP uptake in cultured myotubes (Bursztajn and Libby 1981; Janeczko et al. 1985).

In addition to the vesicular proliferation of the t-system, t-tubuli were frequently oriented longitudinally and appeared to encircle cytoplasmic areas and/or cytoplasmic components. Enlarged t-tubular profiles were also observed in the segments. These processes may also participate in the formation of intracellular HRP-containing bodies.

Segments could already be found in muscles taken 10-15 min after the animal had received an injection of HRP. Only 4 such segments were studied since they were rather difficult to find in the muscles, possibly because they only occurred sporadically or because they did not contain enough HRP to be well stained. Large peroxidase-positive phagosomes could be seen in these segments suggesting that the uptake occurs very rapidly and/or that some large phagosomes or vacuoles are in communication with the extracellular space. Some phagosomes in these and other segments were seen to have "tails" which could suggest a communication with the t-system. However, if a "passive" filling of phagosomes or vacuoles occurs the HRP must be strongly bound inside these structures since it is still present

24 hours after injection. Histochemical studies on denervated hemidiaphragm muscle (results not shown) have confirmed the presence of segments with high HRP uptake 10-15 min after HRP injection as well as at 48 hours after injection. At these times there was more than a 20,000-fold difference in plasma peroxidase activity which suggests that HRP is not completely free to diffuse passively into and out of the segments.

In this context it is interesting to note that the available data on pinocytosis may also be explained without a net translocation of membrane if substances are transferred through a series of compartments transiently interconnected by alternate fusion-fission events or even more permanently interconnected by narrow channels fitted with appropriate locks (Schneider et al. 1979; Lloyd 1980). In rabbit alveolar macrophages, where HRP uptake also occurs by a receptor-mediated process (Kaplan and Nielsen 1978), an ultrastructural study (Nichols 1982) has recently shown that the uptake occurs via smooth tubuli as well as coated vesicles. The events of pinocytosis and digestion in this system were so closely coordinated that sometimes the "outer ends" of the tubuli remained open to the plasmalemma even though their "inner ends" had already merged with lysosomes.

Whatever the detailed mechanism for the HRP uptake is, it seems clear, from the vivid proliferation of the t-system and from the localization of the uptake deep in the muscle fibres rather than immediately under the sarcolemma, that the uptake derives from t-tubuli and that t-tubuli act as a source of membrane for the formation of phagosomes. T-tubuli have previously been reported to act as a membrane source for autophagic vacuoles occurring in muscles of individuals affected by acid maltase deficiency (Engel 1970) and in experimental chloroquine myopathy (MacDonald and Engel 1970; Schmalbruch 1980). In these instances, however, the mechanisms for membrane delivery from the t-system may be different.

Peroxidase-positive phagosomes were often seen to contain undegraded material. Occasionally structures resembling mitochondria could be identified inside such phagosomes. Phagosomes in the process of engulfing cytoplasmic material were also frequently observed. These observations suggest that peroxidase-positive phagosomes participate in autophagic processes, i.e. that they are of lysosomal nature. This is also suggested by the greater accumulation of undegraded material in such phagosomes after chloroquine treatment (Tågerud et al. submitted). The lysosomal nature of at least some of the peroxidase-positive phagosomes is also in agreement with histochemical results showing a similar distribution of peroxidase and acid phosphatase staining in segments with high HRP uptake (Tågerud and Libelius 1984). The presence of undegraded material in peroxidase positive phagosomes

was particularly frequent in fibres taken 10-24 hrs after HRP injection. This indicates that the presence of HRP in phagosomes may interfere with their lysosomal functions.

Large membrane-limited bodies similar to peroxidase-positive phagosomes but with only weak or no peroxidase staining were also observed in the segments. The role of these bodies or their relationship to the phagosomes with high HRP activity is not clear.

Sarcoplasmic reticulum

An overdevelopment and enlargement of the sarcoplasmic reticulum has previously been reported in denervated muscle (Pellegrino and Franzini 1963; Muscatello et al. 1965; Shafiq et al. 1967; Gori 1972; Stonnington and Engel 1973; Tomanek and Lund 1973). In the present study enlarged components of the sarcoplasmic reticulum were frequently encountered in the vicinity of areas with t-tubular proliferation and peroxidase-positive phagosomes. That the enlarged components are derived from the sarcoplasmic reticulum is suggested by their apposition to t-tubuli and by their fine granular appearance which is characteristic of the sarcoplasmic reticulum in material doubly fixed with glutaraldehyde and osmium (Engel and MacDonald 1970). Where t-tubuli were oriented longitudinally the sarcoplasmic reticulum was sometimes still apposed to them as has previously been reported in denervated muscle (Gauthier and Dunn 1973; Cullen and Pluskal 1977).

The function of the enlarged sarcoplasmic reticulum components in the segments is not clear. In dystrophic muscle and in experimental myopathies induced by chloroquine or chlorphentermine it has been suggested that distended portions of the sarcoplasmic reticulum may contain lysosomal enzymes and participate in the formation of autophagic vacuoles (Pearce 1966; Christie and Stoward 1977; Schmalbruch 1980). The presence of acid phosphatase and cathepsin B and D in parts of the sarcoplasmic reticulum of rat and chicken skeletal muscle has been confirmed by cytochemical staining (Pearce 1966; Seiden 1973; Bird et al. 1977; 1978; Trout et al. 1979 a; 1979 b; Spicer et al. 1980). Zonal fractionation studies of lysosome-rich fractions from rat skeletal muscle have also suggested that parts of the sarcoplasmic reticulum contain acid hydrolase activity (Stauber and Bird 1974). It is tempting to speculate that the enlarged components of the sarcoplasmic reticulum are part of the lysosomal system also in the segments described here. This, however, needs to be confirmed by ultra-structural staining for lysosomal enzymes.

Golgi system

Golgi complexes have previously been reported to become more numerous

in denervated muscle (Gori 1972; Tomanek and Lund 1973; Engel and Stonnington 1974; Cullen and Pluskal 1977). In the segments with high HRP uptake an abundance of Golgi profiles were seen, frequently occurring deep in the muscle fibre close to HRP-containing structures. The function of the Golgi system in this context is unclear. In certain myopathies the Golgi system has been shown to contribute membrane to autophagic vacuoles (Engel and Dale 1968, Engel 1970). The Golgi system could also be involved in the sequestering of lysosomal enzymes entering into lysosomes (Cullen and Pluskal 1977), in the recycling of membrane receptors for the endocytic process or in the processing of some substance that may be secreted from the described segments.

Functional significance

From the results presented here and from earlier studies on the uptake of HRP in denervated skeletal muscle (Libelius et al. 1979b; Libelius and Tågerud 1984; Tågerud and Libelius 1984; Tågerud and Libelius 1985) it seems clear that the endplate region of muscle fibres takes on special functions after denervation. Pronounced myofibrillar degeneration occurs in this part of the fibre. Lysosomal enzyme activities are higher here than in surrounding fibre areas. The t-system, sarcoplasmic reticulum and Golgi system all proliferate and a large part of the fibre volume is taken up by large membrane-limited bodies which accumulate glycoproteins rich in mannose residues (such as HRP) from the extracellular space.

The physiological or pathophysiological significance of these changes in the endplate region of denervated muscle fibres is not understood. They may be related to an increased membrane turnover in the muscle fibres after denervation. They could also be involved in nerve-muscle communication by screening the extracellular fluid for presence of "trophic factors" originating from the nerve. Alternatively, these changes in the endplate region of denervated muscle fibres may be involved in muscle-nerve communication by producing and secreting a factor for attracting nerves to the denervated endplate. This would be in accordance with the hypothesis that the denervated endplate is the source of a stimulus to nodal sprouting of motoneurons (Keynes et al. 1983).

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BIOCHEMICAL AND ULTRASTRUCTURAL EFFECTS OF CHLOROQUINE
ON HORSERADISH PEROXIDASE UPTAKE AND LYSOSOMAL ENZYME ACTIVITIES
IN INNERVATED AND DENERVATED MOUSE SKELETAL MUSCLE

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SUMMARY

The effects of chloroquine treatment on horseradish peroxidase (HRP) uptake and lysosomal enzyme activities in innervated and denervated mouse skeletal muscle have been studied using biochemical, histochemical and ultrastructural techniques.

Chloroquine treatment caused a large (59-101%) increase in the activity of cathepsin D in both innervated and denervated muscle. The activity of N-acetyl- β -D-glucosaminidase also increased slightly in denervated muscle. No effect was observed on acid phosphatase activity. The in vivo uptake of HRP in innervated and denervated muscle was unaffected by chloroquine treatment. The results show that the activities of certain lysosomal enzymes may increase in skeletal muscle without an increase in endocytic activity. This is discussed in comparisor to what is seen in denervated and dystrophic muscle.

Histochemical and ultrastructural studies showed the HRP uptake to occur segmentally in denervated muscle fibres from untreated as well as chloroquine treated animals. Ultrastructurally the peroxidase positive phagosomes occurring in these segments were found to contain increased levels of undegraded material after chloroquine treatment suggesting that these phagosomes are of a lysosomal nature and also participate in autophagic processes.

Key words: Chloroquine - Denervation - Endocytosis - Horseradish peroxidase
- Lysosomal enzymes - Myopathy - Skeletal muscle

INTRODUCTION

A number of ultrastructural studies have shown that administration of chloroquine to humans or to experimental animals can produce a myopathy characterized by the presence of lamellar inclusions and autophagic vacuoles in the muscle fibres (Garcin et al. 1964; Rewcastle and Humphrey 1965; MacDonald and Engel 1970; Gerard et al. 1973; Drenckhahn and Lüllmann-Rauch 1979; Lüllmann-Rauch 1979; Schmalbruch 1980; Stauber et al. 1981a; 1981 b; Trout et al. 1981).

In some degenerative muscle disorders, such as denervation and muscular dystrophy, an increased endocytic uptake of extracellular markers has been found to accompany or precede increases in lysosomal enzyme activities and degeneration of muscle fibres (Libelius et al. 1978a; 1978b; 1978c; 1979a; 1979 b; 1981; Thesleff et al. 1979; Sellin et al. 1980; Tågerud and Libelius 1984). The main aim of the present investigation was to study whether or not muscles impaired by chloroquine also show increased uptake of an extracellular marker (horseradish peroxidase, HRP) and/or changes in lysosomal enzyme activities.

Chloroquine is believed to exert many of its actions on cells by interfering with normal lysosomal function (see deDuve et al. 1974; Lüllmann-Rauch 1979). In denervated skeletal muscle an increased uptake of HRP occurs in segments of the muscle fibres which also contain high activities of lysosomal enzymes (Tågerud and Libelius 1984; Libelius and Tågerud 1984) and ultrastructurally HRP is seen to accumulate in large phagosomes which are believed to have lysosomal functions (Libelius et al. 1979 b; Tågerud et al. submitted). A further aim of the present investigation was to study the effects of chloroquine on the ultrastructural appearance of the segments with high HRP uptake in denervated muscle fibres.

MATERIALS AND METHODS

All experiments were performed on the tibialis anterior and extensor digitorum longus muscles of adult male NMRI mice, weighing about 30 g. The animals received an intraperitoneal injection of chloroquine diphosphate 50 mg/kg twice daily for 22 days (only one injection on the last day). This dosage regimen was well tolerated by the animals. Giving the total daily dose in a single injection, however, killed the animals. There was no reduction in weight of the chloroquine treated animals as compared to control animals injected with saline only.

On the 8th day of chloroquine treatment unilateral denervation of the lower hind limb was carried out under diethyl-ether anaesthesia by sectioning

the deep peroneal nerve just above the knee.

On the 22nd day, i.e. after the last chloroquine injection, 20 mg of HRP, dissolved in 0.2 ml of 0.9% saline, were injected into a tail vein 2 hours before killing the animal. Some control animals received only saline injections.

Some ultrastructural studies were also carried out on muscles from animals treated with chloroquine diphosphate 50 mg/kg once daily (except for the first day when two injections were given) for 15 days. Here unilateral denervation was carried out on the second day of chloroquine treatment and HRP was injected on the 15th day as described above.

Biochemical studies

After killing the animal by cervical dislocation the tibialis anterior and extensor digitorum longus (EDL) muscles were dissected, rinsed overnight, weighed, homogenized and assayed for peroxidase, N-acetyl- β -D-glucosaminidase, acid phosphatase and cathepsin D activities as reported previously (Sellin et al. 1980; Tågerud and Libelius 1984).

Protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

Histochemical studies

After killing the animal by cervical dislocation the tibialis anterior muscles were dissected, fixed, frozen, sectioned and stained for peroxidase (Tågerud and Libelius 1984).

Ultrastructural studies

After anaesthetizing the animal by an intraperitoneal injection of pento-barbital fixation of the tibialis anterior muscles and preparation of single muscle fibres containing segments with high peroxidase activity was performed as reported previously (Tågerud et al. submitted).

Statistical evaluation

Statistical significance was tested by means of Student's t-test using unpaired observations.

Chemicals

Special chemicals used were obtained from the following sources: horseradish peroxidase (type II) and tris (hydroxymethyl) aminomethane (Trizma^R base) from Sigma Chemical Co., St. Louis, MO, U.S.A.; 3,3'-diaminobenzidine tetrahydrochloride, haemoglobin (crystalline), and 4-methyl-umbelliferyl-phosphate from British Drug Houses Ltd., Poole, U.K.; glucose oxidase

(grade I) from Boehringer, Mannheim, F.R.G.; glutaraldehyde (8% aqueous, E.M. grade) from Polysciences Inc., Warrington, PA, U.S.A.; chloroquine diphosphate and 4-methyl-umbelliferyl-N-acetyl- β -D-glucosaminide from Serva Feinbiochemica, Heidelberg, F.R.G.

RESULTS

Biochemical studies

The results of the biochemical studies are summarized in Tables I and II for EDL and tibialis anterior muscles respectively. Chloroquine treatment resulted in a tendency towards decreased protein content particularly in denervated muscles although this did not always reach statistical significance.

The HRP uptake was higher in denervated muscle than in innervated muscle as has been reported previously (Libelius et al. 1978b; 1979b; Tågerud and Libelius 1984). An interesting observation which has not been commented upon previously was that the HRP uptake after denervation increased much more in the EDL muscle than in the tibialis anterior muscle. In denervated EDL muscle the HRP uptake was more than 5 times as high as the uptake in innervated muscle. In denervated tibialis anterior muscle, on the other hand, the HRP uptake was not even 3 times as high as in innervated muscle. To a lesser extent this difference between EDL and tibialis anterior muscle could also be seen in the increases in lysosomal enzyme activities after denervation.

Chloroquine treatment did not result in any significant change in the HRP uptake in innervated or denervated muscle. Endogenous peroxidase activity in muscles from animals not injected with HRP was only 1-4% of the total peroxidase activity in muscles from animals injected with HRP. Values given in Tables I and II for HRP uptake have not been corrected for this endogenous peroxidase activity.

Out of the three lysosomal enzymes studied cathepsin D was the only one which showed a large and highly significant increase in activity after chloroquine treatment. This occurred in both innervated and denervated muscle and amounted to a 59-101% increase in activity. The activity of N-acetyl- β -D-glucosaminidase also increased slightly in denervated muscle after chloroquine treatment but this smaller increase reached statistical significance only in the denervated EDL muscle.

Histochemical studies

Transverse sections of denervated tibialis anterior muscle from animals injected with HRP contained fibres with dense peroxidase staining distributed

TABLE I: Effects of chloroquine-treatment on muscle weight, protein content, horseradish peroxidase uptake and lysosomal enzyme activities in innervated and denervated extensor digitorum longus muscle.

Parameters studied	Innervated EDL muscle		Denervated EDL muscle	
	Chloroquine treated	Saline treated	Chloroquine treated	Saline treated
Muscle wet weight (mg)	12.3 $\pm$ 0.4 (11)	12.0 $\pm$ 0.4 (12)	8.1 $\pm$ 0.2 (11)	8.2 $\pm$ 0.2 (12)
Protein content (mg/muscle)	1.63 $\pm$ 0.10(11)	1.74 $\pm$ 0.06 (12)	1.07 $\pm$ 0.03 (11)	1.19 $\pm$ 0.05 (12)
Horseradish peroxidase uptake (μ g HRP/g muscle protein)	186.0 $\pm$ 10.3 (8)	170.5 $\pm$ 4.8 (9)	837.0 $\pm$ 38.0 (8)	874.4 $\pm$ 80.5 (9)
Endogenous peroxidase activity (μ g HRP equivalents/g muscle protein)	6.0 $\pm$ 1.2 (3)	4.7 $\pm$ 0.2 (3)	12.3 $\pm$ 2.8 (3)	9.0 $\pm$ 0.9 (3)
β -N-acetylglucosaminidase activity (μ mol 4-methylumbelliferone liberated/min/g muscle protein)	0.28 $\pm$ 0.01 (11)	0.28 $\pm$ 0.01 (12)	0.78 $\pm$ 0.04 (11)*	0.66 $\pm$ 0.03 (12)
Acid phosphatase activity (μ mol 4-methylumbelliferone liberated/min/g muscle protein)	4.7 $\pm$ 0.2 (11)	4.7 $\pm$ 0.1 (12)	8.8 $\pm$ 0.3 (11)	8.2 $\pm$ 0.2 (12)
Cathepsin D activity (μ mol tyrosine equivalents liberated/min/g muscle protein)	9.2 $\pm$ 0.3 (11)***	5.0 $\pm$ 0.1 (12)	30.3 $\pm$ 1.0 (11)***	15.1 $\pm$ 0.5 (12)

Values given are mean values $\pm$ SEM (n). * p < 0.05; *** p < 0.001.

TABLE II: Effects of chloroquine-treatment on muscle weight, protein content, horseradish peroxidase uptake and lysosomal enzyme activities in innervated and denervated tibialis anterior muscle.

Parameters studied	Innervated tibialis anterior muscle		Denervated tibialis anterior muscle	
	Chloroquine treated	Saline treated	Chloroquine treated	Saline treated
Muscle wet weight (mg)	72.3 [±] 2.4 (11)	72.4 [±] 1.9 (12)	40.8 [±] 1.3 (11)	44.1 [±] 1.3 (12)
Protein content (mg/muscle)	10.5 [±] 0.4 (11)	10.4 [±] 0.3 (12)	5.6 [±] 0.2 (11)*	6.2 [±] 0.2 (12)
Horseradish peroxidase uptake (ug HRP/g muscle protein)	172.1 [±] 20.9 (8)	156.4 [±] 11.9 (9)	407.6 [±] 18.7 (8)	448.1 [±] 43.0 (9)
Endogenous peroxidase activity (ug HRP equivalents/g muscle protein)	5.8 [±] 0.6 (3)	6.1 [±] 1.2 (3)	10.2 [±] 0.7 (3)	9.0 [±] 1.8 (3)
β -N-acetylglucosaminidase activity (umol 4-methylumbelliferone liberated/min/g muscle protein)	0.32 [±] 0.01 (11)	0.33 [±] 0.01 (12)	0.73 [±] 0.04 (11)	0.65 [±] 0.05 (12)
Acid phosphatase activity (umol 4-methylumbelliferone liberated/min/g muscle protein)	5.0 [±] 0.1 (11)	5.2 [±] 0.1 (12)	7.9 [±] 0.3 (11)	7.8 [±] 0.3 (12)
Cathepsin D activity (umol tyrosine equivalents liberated/min/g muscle protein)	8.9 [±] 0.3 (11)***	5.6 [±] 0.2 (12)	25.5 [±] 0.7 (11)***	15.6 [±] 0.5 (12)

Values given are mean values [±] SEM (n). *p < 0.05; *** p < 0.001.

in a ring or occasionally centrally in the fibres as has been described previously (Tågerud and Libelius 1984). Such fibres were also observed in denervated muscles from chloroquine-treated animals injected with HRP (Fig. 1).

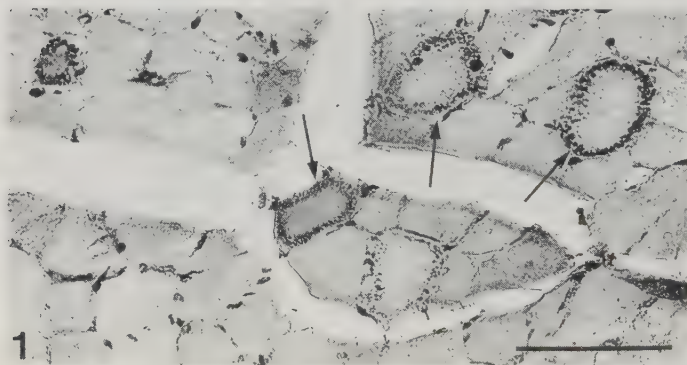


Fig. 1 Transverse section of a 14-days denervated tibialis anterior muscle from an animal treated with chloroquine for 22 days, 2 hrs after HRP injection. Numerous fibres (some denoted by arrows) show HRP uptake with a ring-like distribution. Bar = 100 μ m.

Transverse sections of innervated tibialis anterior muscles from chloroquine treated animals injected with HRP did not show any signs of localized high peroxidase uptake.

Ultrastructural studies

A semithin section containing two fibres from a denervated tibialis anterior muscle after chloroquine treatment and HRP injection is shown in Fig.

2. Only one of the fibres contains a segment with high peroxidase content. The ultrastructural appearance of selected parts of these two fibres is shown in Figs. 3-5. Both fibres contain large chloroquine-induced autophagic vacuoles. In the fibre with the peroxidase containing segment there are also peroxidase positive phagosomes which contain large amounts of undegraded cytoplasmic components. Further examples of such phagosomes are shown in Figs. 6-7. This accumulation of undegraded cytoplasmic material in peroxidase positive phagosomes greatly exceeded that observed in segments from untreated denervated muscle taken 2 hours after HRP injection (Tågerud et al. submitted). Other features of the segments such as the extent of myofibrillar degeneration (Tågerud et al. submitted) appeared to be unaltered by the chloroquine treatment.

Chloroquine-induced autophagic vacuoles and myeloid bodies were observed in segments with high peroxidase content, in other parts of denervated muscle fibres and in innervated fibres. In some muscle fibres from chloroquine-treated animals mitochondria appeared slightly swollen with separated cristae (Fig. 8). This was never observed in muscle fibres from animals not treated with chloroquine.

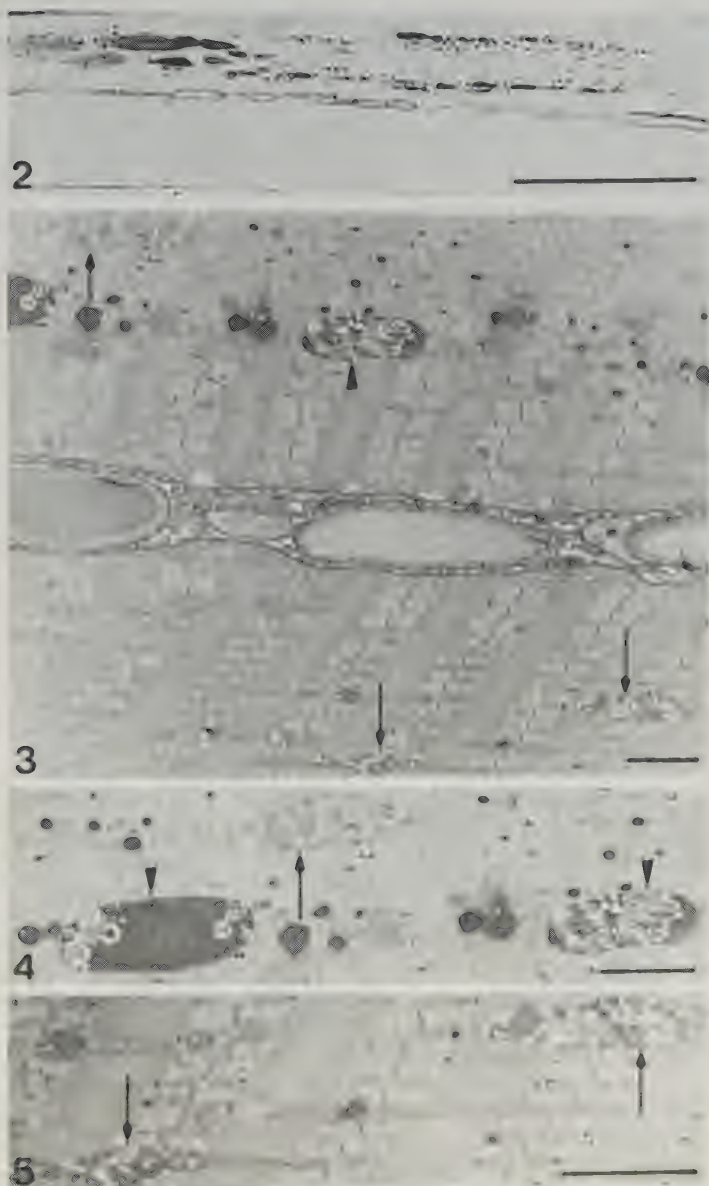
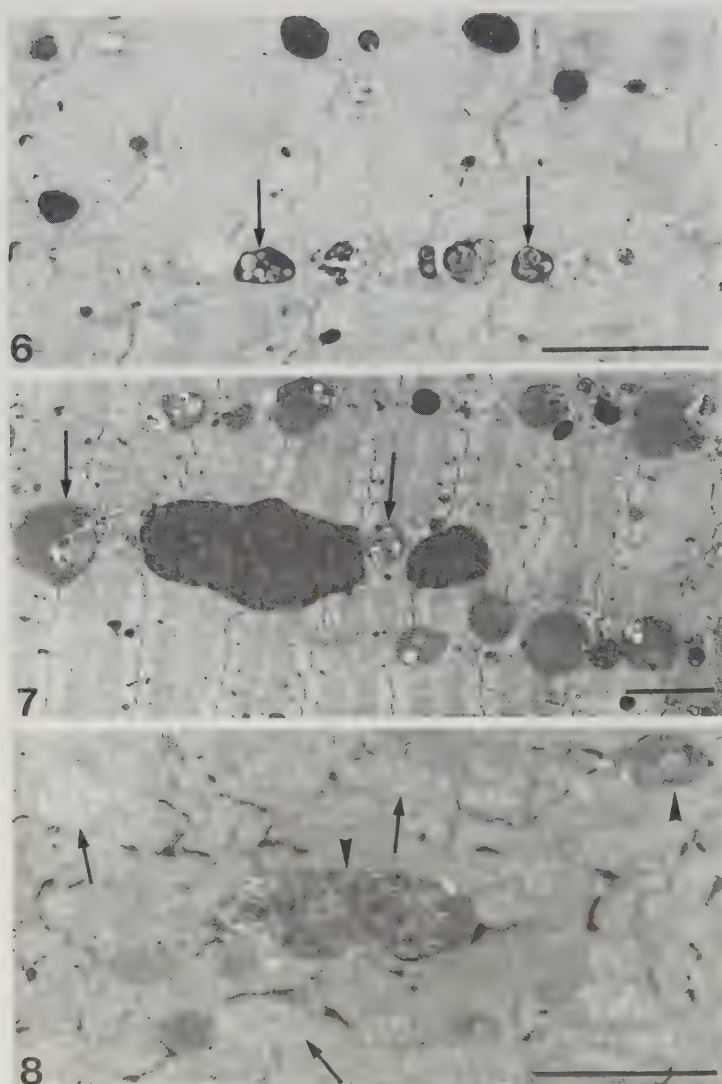


Fig. 2 Semithin section containing two fibres from a 14-days denervated tibialis anterior muscle. 22-days of chloroquine treatment, 2 hrs after HRP injection. Only the top fibre contains a segment with high peroxidase content. Bar = 50 μ m. Figs. 3-5 Ultrastructural appearance of selected parts of the two fibres shown in Fig. 2. Note presence of large chloroquine-induced autophagic vacuoles (arrows) in both fibres. In the fibre with the peroxidase containing segment (Fig. 3 top and Fig. 4) there are also peroxidase positive phagosomes containing undegraded cytoplasmic components (arrowheads). Bars = 2 μ m.



Figs. 6-7 Peroxidase positive phagosomes containing undegraded cytoplasmic material (some denoted by arrows). 14-days denervated, 22-days of chloroquine treatment (Fig. 6) and 13-days denervated, 15-days of chloroquine treatment (Fig.7). 2 hrs after HRP injection. Bars = 2 μ m. Fig. 8 Slightly swollen mitochondria with separated cristae (some denoted by arrows) in a 14-days denervated muscle fibre from an animal treated with chloroquine for 22 days. 2 hrs after HRP injection. Note also presence of chloroquine-induced autophagic vacuoles without prominent peroxidase staining (arrowheads). Bar = 2 μ m.

DISCUSSION

Previous ultrastructural studies have shown that administration of chloroquine to rats or chickens can produce a myopathy characterized by the presence of lamellar inclusions and autophagic vacuoles in the muscle fibres (MacDonald and Engel 1970; Schmalbruch 1980; Stauber et al. 1981a; 1981 b; Trout et al. 1981). In a study on rabbit skeletal muscle, however, chloroquine treatment did not induce the formation of autophagic vacuoles (Aguayo and Hudgson 1970). Hence, there seems to exist species differences in sensitivity to chloroquine. The present study shows that chloroquine can induce ultrastructural changes in mouse skeletal muscle which are similar to those seen in the rat and chicken. Mice may, however, in contrast to rats (MacDonald and Engel 1970; Schmalbruch 1980), be relatively resistant to the effects of chloroquine, since although a rather high dose of chloroquine was given twice daily for 22 days there was no reduction in the weight of the experimental animals as compared to saline-treated controls. The animals moved freely in the cage without any obvious signs of impaired muscle function. Muscle wet weight and muscle protein content were also relatively unaffected by the treatment.

The main aim of the present investigation was to study whether or not the chloroquine-induced myopathy is accompanied by increased endocytic activity and changes in lysosomal enzyme activities. The results indicate that chloroquine treatment has no influence on the uptake of HRP in innervated or denervated muscle. Chloroquine treatment, however, induced a large increase in the activity of cathepsin D in both innervated and denervated muscle. The other lysosomal enzymes studied, acid phosphatase and N-acetyl- β -D-glucosaminidase, showed no or only small changes in activities after chloroquine treatment. The situation after chloroquine treatment is thus quite different from that seen after denervation or in dystrophic muscle where there is both an increase in endocytic activity (HRP uptake) and a more generalized increase in the activities of lysosomal enzymes (see Introduction).

We have previously put forward a hypothesis suggesting that increased endocytosis in skeletal muscle may induce increased lysosomal enzyme activities and muscle fibre degeneration (Jirmanová et al. 1977; Libelius and Lundquist 1978; Libelius et al. 1978b; 1978 c; Thesleff et al. 1979; Thesleff and Libelius 1983). The present results cannot be directly incorporated into this hypothesis since increased endocytosis was not observed after chloroquine treatment. The results show, however, that the activities of certain lysosomal enzymes may increase in skeletal muscle without an increase in endocytic activity. This indicates that several different mechanisms operate in the process of lysosomal activation in skeletal muscle.

The reason for the selective effect of chloroquine treatment on cathepsin D activity is not clear. Another drug, chlorphentermine, which can cause a chloroquine-like myopathy in rats (Drenckhahn and Lüllmann-Rauch 1979; Schmalbruch 1980), has also been shown to increase the activities of some but not all lysosomal enzymes in various rat tissues (Lüllmann and Mössinger 1979). An earlier study of the effect of chloroquine treatment on cathepsin D activity in chicken muscle revealed no change in activity after 7 days of treatment (Stauber et al. 1981a). The discrepancy between the results of that study and the present results may reflect differences in species, dosage and time period of drug treatment.

In vitro chloroquine can inhibit receptor-mediated endocytosis of many ligands (see Shepherd et al. 1983) including the uptake of HRP in innervated and denervated skeletal muscle (Tågerud and Libelius 1985). In cultured myotubes, treatment with chloroquine increases the percentage of coated vesicles labelled with the extracellular marker HRP, suggesting that chloroquine may prevent fusion of lysosomes with coated vesicles originating by endocytosis (Bursztajn and Libby 1981). The results of the present study show that in vivo the HRP uptake is unaltered by chloroquine treatment in both innervated and denervated muscle. This probably reflects the difficulty in attaining adequate concentrations in vivo for the inhibition of uptake to occur.

Another aim of the present study was to study the effects of chloroquine on the ultrastructural appearance of segments with high HRP uptake in denervated muscle. Histochemical staining of denervated muscle fibres or cross sections of denervated muscle revealed no apparent changes in the gross morphological appearance of the HRP-uptake. Ultrastructurally, however, peroxidase positive phagosomes were seen to contain more undegraded material than that usually observed two hours after an HRP injection (Tågerud et al. submitted). Myeloid bodies and autophagic vacuoles without peroxidase labelling were also observed in both innervated and denervated muscle fibres. Chloroquine, by virtue of its physico-chemical properties, accumulates in acidic organelles such as lysosomes and may thereby inhibit their function (see deDuve et al. 1974). It may also, again by virtue of its physico-chemical properties, bind to various phospholipids thereby altering their properties such that they become more resistant to hydrolysis (see Lüllmann-Rauch 1979). These possible alterations in lysosomal function and in the properties of phospholipids may cause the accumulation of myeloid bodies and autophagic vacuoles in muscle fibres. The fact that peroxidase positive phagosomes, present in segments of denervated muscle fibres, contain increased amounts of undegraded material after chloroquine treatment strongly suggests that

these phagosomes are of a lysosomal nature and also participate in autophagic processes.

In some muscle fibres from chloroquine-treated animals mitochondria appeared to be slightly swollen with separated cristae. This is similar to what has previously been reported for chloroquine treated rabbit muscle (Aquayo and Hudgson 1970) and for chloroquine treated foetal mouse hearts in culture (Ridout et al. 1978).

An observation in the present study which has not been commented upon previously is that the HRP uptake increases much more after denervation in the EDL muscle than in the tibialis anterior muscle. To a lesser extent this is also true for the increases in lysosomal enzyme activities after denervation. These differences are not due to differences in muscle atrophy since the tibialis anterior muscle showed a larger decrease in protein content after denervation than did the EDL muscle. Since the HRP uptake occurs only in relatively small segments of the muscle fibres the relationship of the volume of these segments to the total volume of the muscle may be important for the size of the uptake when calculated per mg protein. If the segments are of a similar size in the fibres of different muscles then differences in muscle volume, due mainly to differences in fibre length, will strongly influence the size of the uptake. The same argument may apply also to the activities of lysosomal enzymes since these also increase more in the segments with high HRP uptake than in the rest of the fibres (Libelius and Tågerud 1984). Other factors such as the proportion of red and white fibres in the different muscles may also be of importance. It is also not known whether all denervated fibres exhibit the segmental uptake of HRP to the same extent. If not, varying proportions of fibres engaged in this process may also be of importance for variations in uptake between muscles.

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**EFFECTS OF BOTULINUM TOXIN INDUCED MUSCLE PARALYSIS ON
ENDOCYTOSIS AND LYSOSOMAL ENZYME ACTIVITIES IN MOUSE
SKELETAL MUSCLE**

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ABSTRACT

The effects of botulinum toxin (type A) induced muscle paralysis on endocytosis and lysosomal enzyme activities in skeletal muscle were compared with the effects of surgical denervation. Muscle atrophy, measured as decrease in total muscle protein content, was as large or larger after botulinum toxin treatment as after denervation. Endocytic activity, measured as the *in vitro* uptake of horseradish peroxidase, and the specific activities of the lysosomal enzymes N-acetyl- β -D-glucosaminidase and cathepsin D were all increased six days after denervation. Only the specific activity of cathepsin D was increased six days after botulinum toxin poisoning. The uptake of horseradish peroxidase and the specific activity of N-acetyl- β -D-glucosaminidase were also increased eleven days after poisoning. Transverse sections of eleven days botulinum poisoned muscles from animals injected with horseradish peroxidase showed fibres with dense peroxidase staining similar to those seen in denervated muscle although they seemed to occur less frequently.

The results show that increases in endocytic activity and lysosomal enzyme activities may occur in skeletal muscle without the presence of degenerating axons. The differences in effects of surgical denervation and botulinum toxin induced paralysis are discussed in terms of what is known about the mechanism of action of botulinum toxin and the possible functional roles of the two lysosomal enzymes studied.

Key words: N-Acetyl- β -D-glucosaminidase - Botulinum toxin - Cathepsin D - Denervation - Endocytosis - Horseradish peroxidase - Lysosomal enzymes - Skeletal muscle.

INTRODUCTION

Denervation of skeletal muscle results in increases in endocytic activity (Libelius 1975) and in lysosomal enzyme activities (see Weinstock and Iodice 1969) of the muscle. When horseradish peroxidase (HRP) is used as the extracellular marker the uptake in skeletal muscle has been shown to occur by a mechanism of receptor-mediated endocytosis (Tågerud and Libelius 1985). In denervated muscle the HRP uptake occurs primarily in segments localized at the endplate region of the muscle fibres (Libelius and Tågerud 1984). The increases in lysosomal enzyme activities after denervation occur throughout the length of the muscle fibres but are particularly prominent in the segments where the increased HRP uptake occurs (Libelius and Tågerud 1984; Tågerud and Libelius 1984).

Some of the changes that occur in the properties of skeletal muscle after denervation have been suggested to be triggered by products of nerve degeneration (Vrbová 1967, Cangiano et al. 1984). Lysosomal enzymes in denervated skeletal muscle have also been suggested to derive from degenerating axons at the myoneural junction (Schwartz et al. 1985). It has also been shown, however, that many denervation-like changes, although usually less pronounced than after denervation, can be induced in skeletal muscle by local injection of the neurotoxin produced by *Clostridium botulinum* (see reviews by Sellin 1981; Simpson 1981). Botulinum toxin causes muscle paralysis by inhibiting the evoked release of acetylcholine at the neuromuscular junction (Burgin et al. 1949) and this effect occurs without any changes in the ultrastructural appearance of motor nerve terminals (Thesleff 1960).

The aim of the present investigation was to study the effects of botulinum toxin induced muscle paralysis on HRP uptake and lysosomal enzyme activities in skeletal muscle.

MATERIALS AND METHODS

All experiments were performed on the tibialis anterior (histochemistry) and extensor digitorum longus (biochemistry) muscles of adult male NMRI mice weighing about 30 g. Unilateral denervation was carried out by sectioning the deep peroneal nerve just above the knee, under diethyl-ether anaesthesia. Injections of crystalline *Clostridium botulinum* toxin type A (haemagglutinin + neurotoxin, M.W. ~ 150.000) were made into the anterolateral region of the right hind leg, under diethyl-ether anaesthesia. An amount of the toxin equal to approximately two times the mouse LD₅₀ after intraperitoneal injection, dissolved in 0.05 ml of buffer (Ambache 1949), was injected in a single bolus. To ensure that the muscle paralysis was satisfactory the disability of the animals to spread the toes of the injected leg was checked just before sacrifice. Control animals received an injection of buffer only.

Uptake studies

Animals were sacrificed by cervical dislocation and the extensor digitorum longus muscles were dissected and mounted on perspex holders (6 muscles on each holder) kept in oxygenated Ringer's solution, pH 7.2-7.4 (composition in mM: NaCl 135; KCl 5; CaCl₂ 2; MgCl₂ 1; Na₂HPO₄ 1; NaHCO₃ 15; glucose 11; bubbled with 95% O₂ - 5% CO₂) at room temperature (20-23°C). After mounting, the muscles were placed in 10 ml of an oxygenated incubation solution containing 100 µg/ml of HRP (Sigma, Type II). Incubations were carried out for 2.5 hrs at 37°C. The incubation solution was continuously bubbled with 95% O₂ - 5% CO₂ supplied through a peristaltic pump to give standardized oxygenation and stirring conditions.

After incubation, the muscles were washed overnight in four changes (100-150 ml each) of oxygenated Ringer's solution at 4°C (in a cold room). The muscles were then weighed, stored frozen, homogenized and assayed for peroxidase content as described previously (Tågerud and Libelius 1984).

Lysosomal enzymes

The homogenates of the muscles used for the uptake studies were also assayed for N-acetyl-β-D-glucosaminidase activity and cathepsin D activity as reported previously (Sellin et al. 1980; Tågerud and Libelius 1984).

Protein

Protein was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard.

Statistical evaluation

Statistical significance was tested by means of Student's t-test using unpaired observations.

Histochemical studies

For animals to be used in histochemical studies 20 mg of HRP (Sigma, Type II), dissolved in 0.2 ml of 0.9% saline, was injected into a tail vein 2 hrs before sacrifice. After sacrifice by cervical dislocation the tibialis anterior muscles were dissected, fixed, frozen, sectioned and stained for peroxidase as reported previously (Tågerud and Libelius 1984).

RESULTS

Biochemistry

Extensor digitorum longus muscles were studied 6 and 11 days after denervation or injection of botulinum toxin or buffer. Contralateral muscles from denervated or buffer injected unpoisoned animals were used as controls. Fig. 1A-D summarizes the results of the biochemical studies.

In Fig. 1A the protein content of muscles is shown and this may be taken as a measure of the degree of atrophy occurring after denervation or botulinum toxin poisoning. Six days after treatment the decrease in protein content is similar for denervated and botulinum toxin treated muscles. Eleven days after treatment, however, botulinum toxin treated muscles have a lower protein content than denervated muscles. In botulinum toxin treated muscles the protein content is $59.4 \pm 2.9\%$ (mean $\pm$ S.E.M., $n=11$) of that in buffer treated controls. In denervated muscle the protein content is $69.7 \pm 4.7\%$ (mean $\pm$ S.E.M., $n=6$) of that in untreated controls. The difference between these values does not, however, reach statistical significance ($0.05 < p < 0.10$).

Fig. 1B shows the values for HRP uptake in the muscles expressed per g muscle protein. Denervation results in an increased uptake of HRP already after 6 days. The uptake of HRP after botulinum toxin treatment, however, is not increased until the second time point studied (11 days after treatment). Even at this time point the uptake does not quite reach the size of that seen in denervated muscle, although the difference is not statistically significant. If the HRP uptake is expressed as ng HRP per muscle instead of ng HRP per g muscle protein the uptake 11 days after treatment is 318.4 ± 36.4 (mean $\pm$ S.E.M., $n = 11$) and 210.4 ± 14.8 (mean $\pm$ S.E.M., $n = 6$) for botulinum toxin and buffer treated muscles respectively and 492.7 ± 53.9 (mean $\pm$ S.E.M., $n = 6$) and 155.8 ± 8.4 (mean $\pm$ S.E.M., $n = 12$) for denervated and untreated control muscles respectively. Expressed in this way the difference between botulinum toxin and buffer treated muscles does not reach statistical significance ($0.05 < p < 0.10$) but the uptake in botulinum toxin treated muscles is significantly less than that in denervated muscles ($p < 0.02$).

Fig. 1C shows the specific activity of N-acetyl- β -D-glucosaminidase. The changes seen in the activity of this lysosomal enzyme resemble the changes seen in HRP uptake at the different times after denervation or botulinum toxin poisoning.

The specific activity of cathepsin D is shown in Fig. 1D. This lysosomal enzyme behaves differently from N-acetyl- β -D-glucosaminidase in that the specific activity is increased already 6 days after botulinum toxin poisoning which is similar to what is seen following denervation. The increase at this time point is significantly ($p < 0.05$) smaller than that seen in the denervated muscles if activity is expressed per g muscle protein but not if activity is expressed as total activity per muscle.



Fig. 1 Muscle protein content (A), endocytic activity (B) and lysosomal enzyme activities (C and D) in control (C, ▨) extensor digitorum longus muscles and six and eleven days after denervation (Den, □), botulinum toxin (BoTx, ■) or buffer (Buff, ▩) treatment. Mean values $\pm$ S.E.M. (number of observations given in each column). ** $p < 0.01$; *** $p < 0.001$.

Histochemistry

Transverse sections of denervated tibialis anterior muscle from animals injected with HRP contain fibres with dense peroxidase staining usually distributed in a ring-like fashion in the fibres (Fig. 2A). Such fibres were also observed in transverse sections of 11 days botulinum toxin poisoned muscles from animals injected with HRP (Fig. 2B). No such fibres were observed in normal innervated or buffer treated muscles from animals injected with HRP.

No attempt was made to quantitatively compare the number of fibres with dense peroxidase staining in botulinum toxin poisoned and denervated muscles. The general impression was, however, that such fibres occurred less frequently in botulinum toxin poisoned muscles than in denervated muscles.

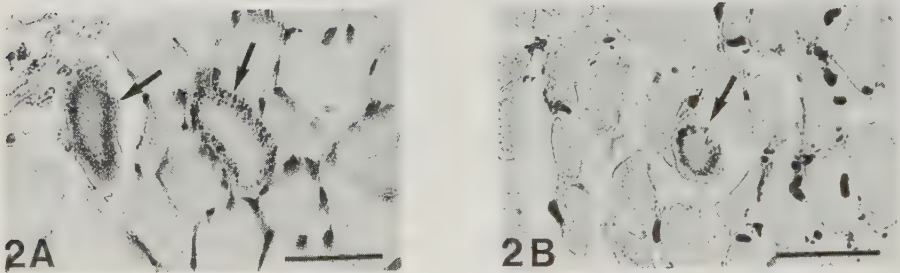


Fig. 2 Transverse sections of fixed 11 days denervated (A) or botulinum toxin treated (B) tibialis anterior muscles taken 2 hrs after an intravenous injection of horseradish peroxidase. Arrows denote fibres showing HRP uptake with a ring-like distribution. Bars = 50 μ m.

DISCUSSION

The results presented here show that botulinum toxin induced muscle paralysis causes qualitatively similar changes in endocytosis and lysosomal enzyme activities in skeletal muscle as does denervation. Some quantitative differences between the changes after botulinum toxin and denervation were, however, observed.

Six days after toxin injection there is no increase in the uptake of HRP whereas after surgical denervation the endocytic activity of muscle increases already 3-4 days after nerve section (Sellin et al. 1980; Tågerud and Libelius 1984). Eleven days after treatment, however, the HRP uptake is increased in muscle paralyzed by botulinum toxin as well as in denervated muscle, although the uptake after botulinum toxin poisoning is smaller than that after denervation. The increase in HRP uptake caused by botulinum toxin induced paralysis thus appears with a slower time course than after denervation and the increase is also smaller in magnitude, at least for the time period (11 days) included in this study.

Transverse sections of 11 days botulinum toxin poisoned tibialis anterior muscle from mice injected with HRP also reveal the presence of fibres with strong peroxidase staining similar in appearance to those seen after denervation (Tågerud and Libelius 1984). In the histochemical study no exact quantitation of the number of fibres with high peroxidase staining was performed. However, the general impression was that in botulinum toxin paralyzed muscle there are less such fibres than in denervated muscle.

The reason for the smaller increase in HRP uptake after botulinum toxin paralysis is not clear. Botulinum toxin prevents the evoked release of acetylcholine (Burgess et al. 1949) and reduces the molecular leakage of acetylcholine from nerve terminals (Polak et al. 1981; Doležal et al. 1983). Despite this the nerve clearly retains some influence over the muscle since botulinum toxin fails to affect the morphological differentiation of the postsynaptic part of the neuromuscular junction following bupivacaine induced degeneration-regeneration of muscle fibres (Jirmanová and Thesleff 1976). Similarly, many other denervation-like changes, such as the increase in number of extrajunctional acetylcholine receptors (Pestronk et al. 1976; Mathers and Thesleff 1978) and the appearance of tetrodotoxin-resistant action potentials (Mathers and Thesleff 1978), are not as marked after botulinum toxin poisoning as after denervation. The exact nature of the remaining influence of the nerve which is responsible for preventing full scale denervation effects is still unknown.

The activities of the two lysosomal enzymes studied, N-acetyl- β -D-glucosaminidase and cathepsin D, react differently in muscles paralyzed with botulinum toxin. N-acetyl- β -D-glucosaminidase is a lysosomal enzyme which participates in the degradation of the glycan part of glycoproteins (see Gregoriadis 1975) and its activity in muscle changes after botulinum toxin induced paralysis in a way similar to the changes seen in the uptake of the glycoprotein HRP. Both the HRP uptake and the specific activity of N-acetyl- β -D-glucosaminidase are unchanged 6 days after botulinum toxin poisoning and both are increased at 11 days after poisoning. Cathepsin D is a lysosomal enzyme involved in the degradation of proteins and is able to degrade both

purified actin and myosin as well as the actin and myosin contained in the structural configuration of myofilaments and myofibrils (Bird et al. 1977; Schwartz and Bird 1977). The enzyme has been implicated to play a role in conditions of muscle wasting (see Weinstock and Iodice 1969; Bird et al. 1978; 1980; Millward 1980). In the present study the activity of cathepsin D reacted to botulinum toxin induced muscle paralysis in a way similar to what was seen for this enzyme after surgical denervation. In this context it is interesting to note that botulinum toxin treatment results in a loss of muscle protein content which is as large, or larger than that seen after surgical denervation.

With regard to the potential contribution of nerve degradation products to the appearance of denervation-like changes (see INTRODUCTION) the present study shows that increases in endocytic activity and lysosomal enzyme activities may occur in skeletal muscle without the presence of degenerating axons.

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